# THE STUDY OF HISTOLOGICAL GRADING BY MODIFIED BLOOM RICHARDSON GRADING SYSTEM AND ER, PR, HER-2/neu STATUS IN INVASIVE BREAST CARCINOMA BY DIAGNOSTIC IMMUNOHISTOCHEMISTRY USING TISSUE MICROARRAY

**DISSERTATION**

**SUBMITTED TO THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY**

**CHENNAI**

**In partial fulfillment of**
**the requirements for the degree of**

**M.D. (PATHOLOGY)**

**BRANCH – III**


**DEPARTMENT OF PATHOLOGY**

**TIRUNELVELI MEDICAL COLLEGE HOSPITAL**

**TIRUNELVELI - 627011**

**APRIL-2016**

# CERTIFICATE

I hereby certify that this dissertation entitled **"THE STUDY OF HISTOLOGICAL GRADING BY MODIFIED BLOOM RICHARDSON GRADING SYSTEM AND ER, PR, HER-2/neu STATUS IN INVASIVE BREAST CARCINOMA BY DIAGNOSTIC IMMUNOHISTOCHEMISTRY USING TISSUE MICROARRAY"** is a record of work done by **Dr. MANORANJANI. R**, in the Department of Pathology, Tirunelveli Medical College, Tirunelveli, during her postgraduate degree course period from 2013-2016. This work has not formed the basis for previous award of any degree.

**The DEAN**

Tirunelveli Medical College,

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DESIGNATION OF PRINCIPAL INVESTIGATOR: POST GRADUATE IN PATHOLOGY
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*Dear ,Dr. R. Manoranjani, MBBS,., The Tirunelveli Medical College Institutional Ethics Committee (TIREC) reviewed and discussed your application during the IEC meeting held on 27.12.2013.*

**THE FOLLOWING DOCUMENTS WERE REVIEWED AND APPROVED**

1. TIREC Application Form ☐
2. Study Protocol ☐
3. Department Research Committee Approval ☐
4. Patient Information Document and Consent Form in English and Vernacular Language ☐
5. Investigator's Brochure ☐
6. Proposed Methods for Patient Accrual Proposed ☐
7. Curriculum Vitae of the Principal Investigator ☐
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10. Investigator's Undertaking ☐
11. DCGI/DGFT approval ☐
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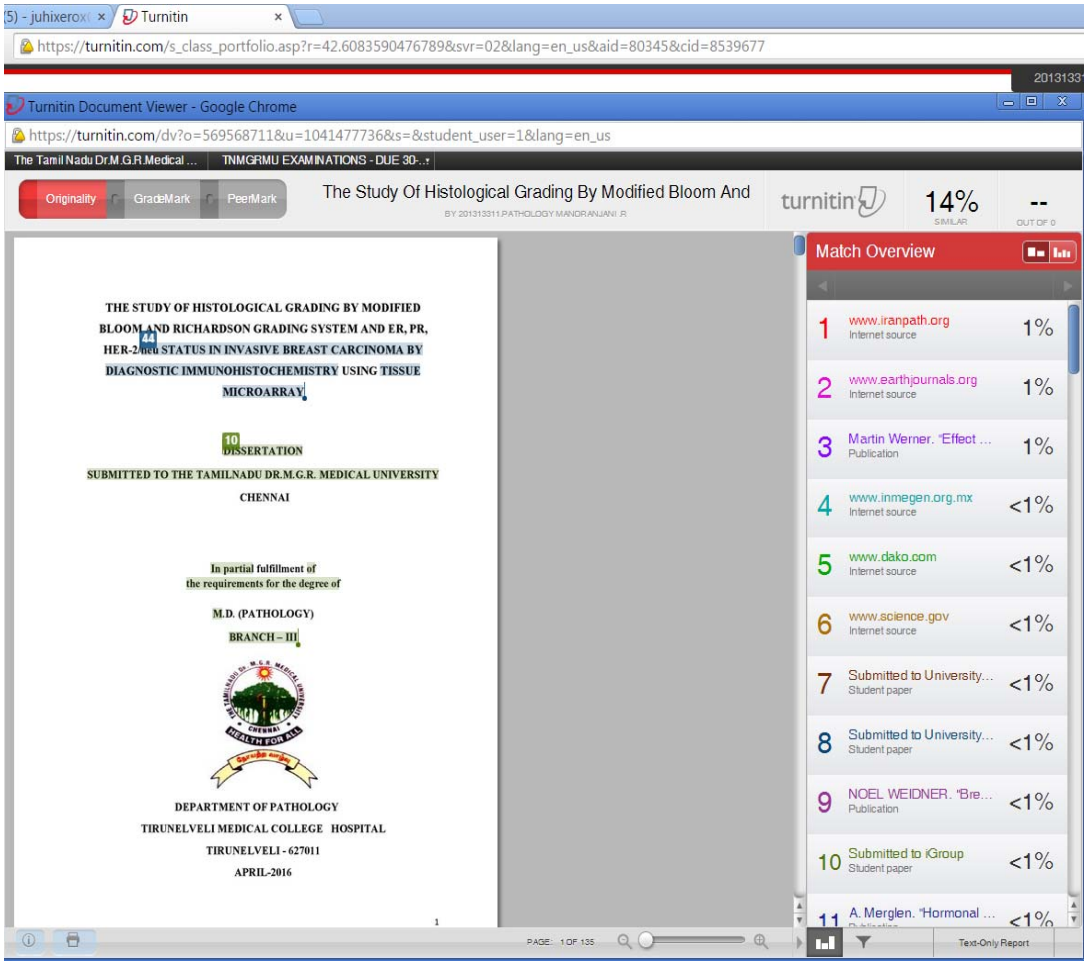
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# DECLARATION

I solemnly declare that this dissertation titled **"THE STUDY OF HISTOLOGICAL GRADING BY MODIFIED BLOOM RICHARDSON GRADING SYSTEM AND ER, PR, HER-2/neu STATUS IN INVASIVE BREAST CARCINOMA BY DIAGNOSTIC IMMUNOHISTOCHEMISTRY USING TISSUE MICROARRAY"** submitted by me for the degree of M.D, is the record work carried out by me during the period of 2013-2016 under the guidance of **Prof. Dr. ARASI RAJESH, M.D**, Professor of Pathology, Department of Pathology, Tirunelveli Medical College, Tirunelveli. The dissertation is submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai, towards the partial fulfilment of requirements for the award of M.D. Degree (Branch III) Pathology examination to be held in April 2016.

Place: Tirunelveli
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# ABBREVIATIONS

| | | |
|---|---|---|
| 1. | DCIS | Ductal Carcinoma In Situ |
| 2. | IDC | Invasive Ductal Carcinoma |
| 3. | IDC, NOS | Invasive Ductal carcinoma, Not otherwise specified |
| 4. | ICC | Invasive Cribriform carcinoma |
| 5. | MBR | Modified Bloom and Richardson |
| 6. | ASCO | American Society Of Oncology |
| 7. | CAP | College of American pathologists |
| 8. | ER | Estrogen Receptor |
| 9. | PR | Progesterone Receptor |
| 10. | HER2/neu | Human Epidermal Growth Factor/neuroblastoma |
| 11. | CK | Cytokeratin |
| 12. | GCDFP | Gross cystic disease fluid protein |
| 13. | BRCA | Breast cancer |
| 14. | EGFR | Epidermal growth factor receptor |
| 15. | IHC | Immunohistochemistry |
| 16. | HRP | Horse Radish Polymer |
| 17. | TRIS – EDTA | Trizma base ( Tris – hydroxyl methyl aminomethane) – Ethylene diamine tetra acetic acid |
| 18. | TMA | Tissue Microarray |
| 20. | +, + ve | Positive |
| 21. | -, - ve | Negative |

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## ABSTRACT

**BACKGROUND:** Breast carcinoma, the most common malignant tumour among women contributes to a significant proportion of all cancers in women worldwide. It was graded based on levels of nuclear pleomorphism, tubular formation and mitotic index. The commonly used predictive immunohistochemical markers are estrogen receptors, progesterone receptors and HER2/neu status. In our study we use microarray technique where small representative tissue samples from many cases were assembled on a single histology slide and subjected to Immunohistochemical analysis.

**AIMS AND OBJECTIVES:** To study histological grading by Modified Bloom Richardson grading system and ER, PR & HER 2/ neu status in invasive breast carcinoma by diagnostic immunohistochemistry using Manual Tissue Microarray and assess the cost-effectiveness of IHC done on TMA slides.

**MATERIALS AND METHODS:** 50 cases of invasive breast carcinoma were included in the study. Histopathological examination of the haematoxylin and eosin stained slides were done and the tumour was graded according to Modified Bloom Richardson grading system. A standard method of microarray preparation was done. First the design for TMA construction was laid out. Paraffin embedded tissue blocks were collected and the areas of invasive carcinoma were cored from donor blocks and transferred to the recipient blocks using bone marrow needle. Thus tissue microarray was constructed manually. Immunohistochemical analysis using ER, PR and Her2/neu were done for all these cases. Evaluation was done with Allred scoring system for ER and PR status, and ASCO guidelines for HER2/neu status.

**RESULTS:** Of the 50 patients analysed, majority were invasive ductal carcinoma (84%). Majority of the invasive breast carcinoma were of MBR grade II (50%) followed by grade III tumors (42%) and grade I tumors (8%). Among 50 cases, ER and PR were positive in 24 cases (48%) and 31 cases (62%) respectively. HER-2/neu expression was seen in 25 cases (50%). A statistically significant correlation was noted between histologic grading and ER, PR and HER2/neu status. The tissue microarray uses only one seventh of the reagent consumed by conventional immunohistochemistry.

**CONCLUSION:** The process of immunohistochemistry using tissue microarray obviates the need for control and standardisation. This allows the study of different cases on a single slide, thus reducing the amount of reagent, duration and labour of the procedure and making it cost effective. Tissue loss due to technical problems can be overcome by following standard protocols or by obtaining more number of tissue cores.



**KEYWORDS:** Breast carcinoma, Modified Bloom Richardson Grading, Immunohistochemistry, Tissue microarray.

# INTRODUCTION

Breast carcinoma is the most common malignant tumor among women. It contributes to a significant proportion of all cancers in women worldwide (25%). Annually about one million women are diagnosed with breast cancer worldwide[1]. Breast cancer accounts for maximum number of deaths in the age group of 15-54 years[2].

There is an increased trend in the detection of breast carcinoma, which can be attributed to increased mammographic screening and changes in lifestyle[3]. But the mortality has decreased due to early screening, which detects the tumor at an early curable stage and also by means of better effective treatment modalities. Nowadays the incidence of breast carcinoma has increased in less developed countries owing to gradual changes in lifestyle of women.

Breast cancer is a heterogeneous disease with distinct biological subtypes. Major types includes invasive ductal carcinoma and invasive lobular carcinoma. Of these, invasive ductal carcinoma is the most common subtype accounting for 70-80%, it is further sub classified as well differentiated (grade1), moderately differentiated (grade2) and poorly differentiated (grade3) based on levels of nuclear pleomorphism, tubular formation and mitotic index[4].

The prognostic factors include clinical variables like age, menopausal status etc., and pathological variables like tumor size, histological type, histological grade and lymph node status[5].

In addition to this there are certain predictive factors like[6],

1. Estrogen and progesterone receptor(ER, PR).
2. HER2/neu amplification.
3. Proliferative markers like Ki-67

The outcome of the tumor varies in each individual and is believed to be due to the heterogeneous nature of the tumor.

Tissue microarray is a recent innovation in the field of pathology. A microarray contains many small representative tissue samples from hundreds of different cases assembled on a single histologic slide and therefore allows high throughput analysis of multiple specimens at the same time, this can be subjected to Immunohistochemistry for analysis of carcinoma[7]. This study is based on Histologic grading and Immunohistochemical analysis of ER, PR & HER 2/ neu status in invasive breast carcinoma for 50 cases using TMA. A major setback of IHC procedure is high cost of monoclonal antibodies and their limited shelf life which may be overcome by TMA.

**AIM:**

To study histologic grading by Modified Bloom Richardson grading system and ER, PR & HER 2/ neu status in invasive breast carcinoma by diagnostic Immunohistochemistry using Manual Tissue Microarray.

**OBJECTIVES:**

- To grade Invasive Breast carcinoma by Modified Bloom Richardson grading system.
- To apply a panel of IHC markers on Invasive breast carcinoma.
- To assess the time and cost effectiveness of IHC done on TMA slides.
- To standardize the procedure of manual TMA.

# REVIEW OF LITERATURE

## HISTORY:

The first description of breast cancer dates back more than 3500 years. The Egyptians were the first to describe breast cancer as bulging tumor in breast .The descriptions of Edwin Smith and George Ebers Papyrus about breast tumors match with the present day scientific descriptions of breast cancer. The etiology of breast cancer was a matter of great speculation. Hippocrates was the first to describe breast cancer as a humoral disease and also named cancer as karkinos which is a Greek word for crab[8].

## EMBRYOLOGY

The breast is a modified apocrine sweat gland and forms an important accessory organ of female reproductive system.

The mammary glands develop at the fifth week of fetal development from the ectodermal mammary ridges which are present on the ventral surface of the fetus bilaterally from the axillary to the inguinal region. During the normal course of development majority of the mammary ridge disappears at the seventh week of gestation.

A small portion of the mammary ridge persists as primary mammary buds which are present in fourth or fifth intercostal spaces. Development proceeds by the penetration of mesoderm by the primary buds of ectoderm. Eventually the

primary mammary buds develop into secondary buds at around 3rd month of gestation and the primary bud later contributes to mammary lobules.

At 20 weeks of gestation, the developing breast is penetrated by multiple radial ingrowths of ectoderm. This is followed by the canalization of the buds which are the precursors of the lactiferous ducts and their branches. The mammary pit which is formed during gestation by the convergence of the lactiferous ducts, then transforms into nipple during infancy.

In males, there is no significant postnatal development of the breast but in females during puberty under the influence of sex hormones the parenchyma and the ductal system proliferates rapidly[9].

**ANATOMY**

The breast is covered by skin and subcutaneous tissue and it lies on the pectoral muscle which is separated by a fascia. It extends from the 2nd to 6th rib vertically and from the lateral border of sternum to the mid axillary line horizontally. A small extension called axillary tail of spence extends laterally towards the axilla.

The nipple lies at the level of 4th intercostal space and is pierced by 15-20 lactiferous ducts. Surrounding the nipple is the areola which is a circular pigmented area and is rich in modified sebaceous glands. Fibrous strands extend from the dermis into the breast, which attach the skin and nipple to the breast called the suspensory ligaments of Cooper.

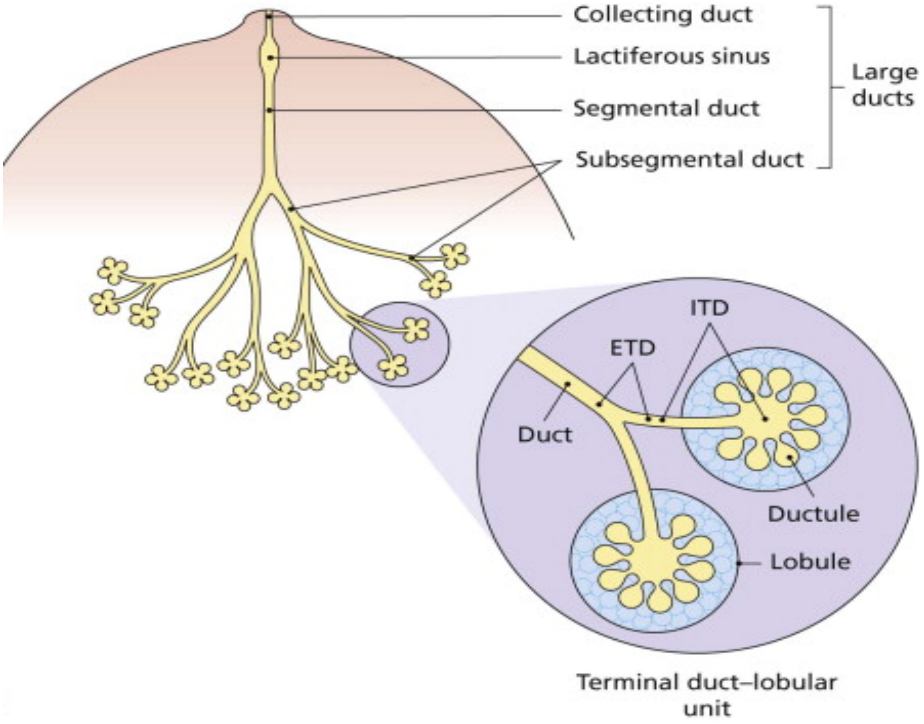



**Fig 1(A)-Diagramatic representation of Terminal Ducto-lobular unit**

The breast parenchyma is composed of glandular tissue which is arranged topographically into lobes. The lobes are made up of terminal duct lobular unit [TDLU] and the large duct system. The TDLU is the secretory portion of breast and consists of lobule and terminal ductule. Each lobule in turn is a cluster of acini. The TDLU connects with the lactiferous (collecting) duct by means of sub segmental and segmental duct. The lactiferous duct opens in to the nipple. A fusiform dilatation called the lactiferous sinus is present between the lactiferous and segmental duct [Fig 1(A)] [(10)].

The intralobular stroma appears myxoid and envelopes the acini of the lobules and consists of breast-specific hormonally responsive fibroblast-like

cells admixed with scattered lymphocytes. The interlobular stroma is made of dense fibrous connective tissue admixed with adipose tissue[10].

**BLOOD SUPPLY**

Arterial supply of breast is by,

i. Internal thoracic artery.
ii. Branches of the lateral thoracic, superior thoracic and acromiothoracic arteries.
iii. Lateral branches of posterior intercostal arteries.

Venous drainage of the breast follows the course of arteries forming an anastomotic circle in the subcutaneous tissue beneath the nipple-areola complex.

From this the veins run as,

1. Superficial veins draining into internal thoracic vein.
2. Deep vein draining into internal thoracic, axillary and posterior intercostal veins[10].

**NERVE SUPPLY:**

Nerve supply is by anterior and lateral cutaneous branches of $4^{th}$ and $6^{th}$ intercostal nerves[10].

## LYMPHATIC DRAINAGE

1. Axillary lymph nodes: Lymphatic drainage is mainly into the anterior group of axillary nodes. Posterior, lateral, central and apical groups of nodes also receive lymphatic drainage either directly or indirectly.
2. The internal mammary nodes which lies along internal thoracic vessels.
3. Supraclavicular node, cephalic node, posterior intercostal, Sub diaphragmatic and sub peritoneal lymph plexus[10].
4. The superficial lymphatics drains overlying skin of breast except nipple and areola .They pass radially to the surrounding lymph nodes (axillary, internal mammary, supraclavicular and cephalic node ).
5. The deep lymphatics drain the parenchyma, nipple and areola of breast. About 75% of lymph drains into axillary nodes, 20% into internal mammary nodes and 5 % into posterior intercostal nodes[10].

## HISTOLOGY

The nipple and the skin are lined by the keratinizing stratified squamous epithelium. The entire ductal-lobular unit lies on a continuous basement membrane. It is lined by two cell layers: luminal epithelial cells and basally located myoepithelial cells [Fig 1(B)]. Luminal cells are either columnar or cuboidal depending on their function. Other cell types present in the breast include scattered endocrine cells[10].

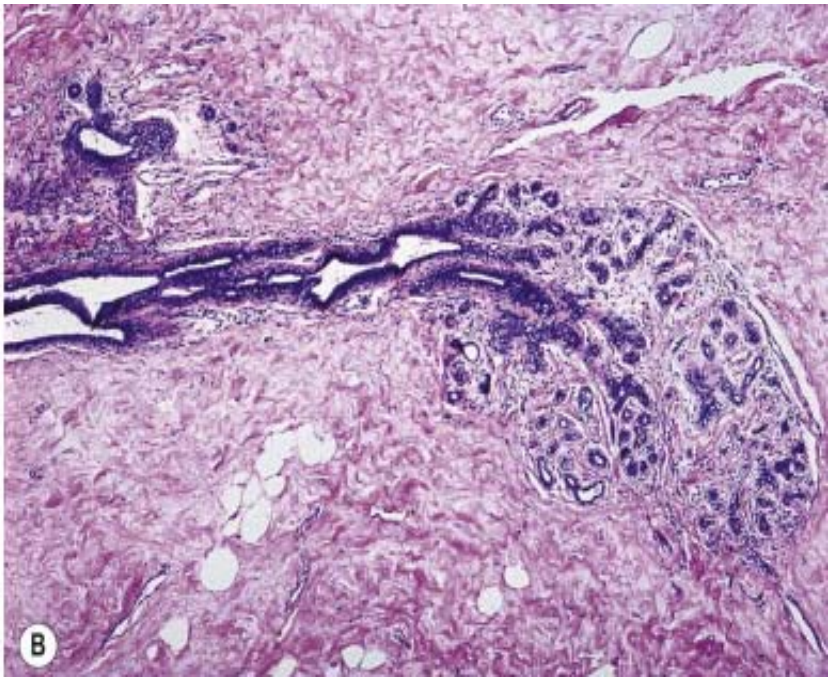



**Fig. 1(B) - Photomicrograph of Terminal Ducto-lobular unit in a normal adult female (H & E).**

The nipple is formed by the lactiferous duct along with the sebaceous unit. The main difference of epidermis of nipple and areola is the increased melanin content in basal layer compared to the normal skin. The basal layer also contains occasional Toker cells[10].

Lactatory function of the breast is a co-ordinated effort of the lobular and myoepithelial cells. The secretory function is by the luminal cells in the lobules. The ejection of milk is by the contractile myoepithelial cells which also renders a structural support to lobules[10].

When analyzed immunohistochemically the luminal epithelial cells stain positive for keratin, EMA, lactalbumin, GCDFP-15. Myoepithelial cells are

positive for S-100, Smooth Musle Actin, calponin, caldesmon (duct portion) and also shows nuclear reactivity for p63[10].

## CARCINOMA BREAST

Breast cancer is the most common malignant cancer among women[1]. The incidence has increased nowadays due to increasing awareness of the people and use of diagnostic modalities like mammography, fine needle aspiration and core biopsy[3].

## EPIDEMIOLOGY

Breast cancer is one of the most prevalent diseases affecting women. Observation is that the incidence of breast cancer has increased globally over the last several decades[11]. And the more worrisome fact is that the greatest increase has been in Asian countries[12]. Worldwide, breast cancer is the most frequent cancer in women and represents the second leading cause of cancer death among women (after lung cancer)[13,14]. The number of new cases occurring each year is estimated to be around 1,00,000[15].

Breast cancer though a global disease does not have a uniform epidemiological pattern throughout. The age group of women affected varies like in Asia, breast cancer incidence peaks among women in their forties, whereas in the United States and Europe, it peaks among women in their sixties. Due to the early onset of disease in our part of the world, premenopausal patients constitute a larger proportion compared to western data[16]. Even in India, breast

cancer is the commonest cancer in women in cities whereas in rural areas, it is second to cervical cancer.

## CLINICAL PRESENTATION

Breast cancer can present in many different patterns. The most common symptoms are breast lump (60-70%) followed by pain (14-18%).Nipple discharge (7-9%) is the least common presenting symptom. With the introduction of mammographic screening there is an increased detection of asymptomatic cases. Anatomically upper outer quadrant contributes to majority of cases (40-50%) followed by central, upper inner, lower outer to lower inner quadrant[17].

Breast mass should be evaluated by triple assessment which includes clinical examination, imaging studies (mammography, ultrasound) and tissue sampling either by fine needle aspiration cytology or core needle biopsy.[17]

## MICROSCOPIC TYPES

1. Carcinoma In Situ
2. Invasive Carcinoma

## INVASIVE DUCTAL CARCINOMA

Whenever a tumor of breast shows a stromal invasion, it is described as invasive ductal carcinoma. Regardless of the presence of in situ component and the relative proportion of in situ and invasive component they are included under invasive carcinoma[10].

Two major categories of invasive carcinoma are - ductal and lobular type. Invasive ductal carcinoma comprises 75-85% of mammary carcinoma. Invasive ductal carcinoma, not otherwise specified comprises majority of duct carcinoma. Other relatively infrequent forms of infiltrating ductal carcinoma include tubular, medullary, metaplastic, colloid carcinoma etc[18].

## CYTOARCHITECTURAL TYPES

### 1. INVASIVE DUCTAL CARCINOMA, NOS TYPE

The major chunk of all breast carcinomas is comprised by IDC, NOS type (75 %). and is considered as the prototype of all breast carcinomas[19].

### GROSS:

The tumor is usually an ill circumscribed firm tumor. Cut section reveals a yellowish gray cut surface with multiple trabeculae radiating through the surrounding parenchyma in to the adjacent fat with a crab like or stellate configuration. In case of larger tumors, areas of necrosis, hemorrhage and cystic degeneration may be present. Scirrhous carcinoma was the term used synonymously with these tumors as they are hard in consistency due to large amounts of stroma.

### MICROSCOPY:

The tumor shows various growth patterns like diffuse sheets, well defined nests, cords, trabeculae and also as individual cells. Glandular differentiation of the tumor varies from almost nil to well developed. The characteristic tumor

cells are usually large and pleomorphic. The tumor shows standard features of malignancy like prominent nuclei and nucleoli and increased mitotic figures. About 60% of the cases show areas of necrosis and calcification. The amount of stroma varies from scant to abundant desmoplastic stroma. The interphase between the tumor and stroma shows mononuclear cell inflammatory infiltrates[10].

Fisher et al. noted lymphatic, blood vessel and perineural invasion in 33%, 5% and 28% of the cases respectively[20].

**IHC:**

The tumor cells are positive for ER, PR, HER2/neu, low molecular weight keratin (8, 18 and 19) and EMA. Other sensitive breast related markers are mammoglobin and GCDFP 15.The basement membrane components collagen 4 and laminin shows a discontinuous linear pattern or it may be totally absent[10].

**2. INVASIVE CRIBRIFORM CARCINOMA**

Invasive cribriform carcinoma is a rare form of breast malignancy which has an excellent prognosis.

**MICROSCOPY:**

The tumor shows a cribriform appearance similar to that of its intraductal counterpart but in addition it shows stromal invasion. Cribriform pattern is often seen in association with tubular formations. Page et al proposed that the relative proportion of the two elements determine the term to be used[21].

## 3. TUBULAR CARCINOMA

Pure tubular carcinoma contributes only a small portion of invasive breast cancer. But in mammographic screening 9-19 % of cases are noted as speculate nature and cellular stroma which is characteristic of this tumor can be easily noted.

**GROSS:**

In gross section the tumor appears poorly circumscribed and is hard in consistency and the size of the tumor is usually small with a mean diameter of 1 cm.

**MICROSCOPY:**

The characteristic feature of tubular carcinoma is the irregular and angulated contour of the glands which shows no organoid configuration. The lining cells show apocrine type snouts in the apical cytoplasm. This tumor typically lacks myoepithelial cells and basement membrane. The lumina of the glands are open and filled with basophilic secretion. The tumor shows cellular desmoplastic reaction with fat invasion in the periphery. It can confused with benign disorders because of the well differentiated nature of the glands, scant pleomorphism and absence of necrosis[(10)]. DCIS can be seen in majority of the cases. The in situ component is usually of low grade showing cribriform or papillary pattern.

Since the tubular pattern in histopathology can be seen associated with invasive ductal carcinoma, NOS type or sometimes with invasive lobular

carcinoma there can be a diagnostic dilemma. In such instances, the term tubular NOS and tubular mixed can be employed. The term tubular carcinoma can be best employed for tumors in which tubular pattern is present for at least 90 % of the tumor. These tumors are associated with favorable prognosis

**4. MUCINOUS CARCINOMA**

Mucinous carcinoma was classified under mucin producing carcinoma. The age groups usually affected by this tumor are postmenopausal women. It is also called as mucoid, gelatinous or colloid carcinoma[17].

**GROSS:**

The tumor is well circumscribed .Cut surface of the tumor shows a characteristic glistening and gelatinous appearance.

**MICROSCOPY:**

The tumor cells are arranged usually in small clusters floating in a mucinous pool which are surrounded by bands of fibrous septa. The malignant cells are characterized by little pleomorphism and a low mitotic rate. The mucin is usually extracellular.

Histochemically the mucins are o-acylated forms of sialomucin. Immunohistochemically there is strong MUC2 positivity in cytoplasm. Estrogen and progesterone receptors are always positive whereas HER2/ neu will be negative[22].

Two different types of mucinous carcinoma are seen based on the endocrine differentiation. Type A tumors shows trabeculae of malignant cells with minimal intracytoplasmic mucin. The cells are conspicuous by their absence of argyrophilia. Type B tumor shows sheets of tumor cells with abundant intracytoplasmic mucin with argyrophilia.

Nodal metastasis is very low which accounts for 2-4% of all cases.

**IHC:**

They are positive for estrogen and progesterone receptors. They usually do not show HER2/neu overexpression or p53 accumulation.

**4. MEDULLARY CARCINOMA**

The tumor is most common in patients under 50 years of age and is common among carriers of BRCA1 mutation.

**GROSS:**

The tumor is well circumscribed, solid and homogenous.

**MICROSCOPY:**

The tumor grows in a diffuse pattern with minimal or absent glandular differentiation. The individual tumor cells are large, pleomorphic with large nuclei and prominent nucleoli. The distinct features of this tumor in microscopy are prominent lymphoplasmacytic infiltrate at the periphery of the tumor. The infiltrate was thought to be due to the reaction of host tissues to the neoplasm.

**IHC:**

They are positive for CK, p53 and negative for hormone receptors (ER, PR), Her2/neu and come under triple negative phenotype. The tumor expresses HLA-DR antigen which could be the possible reason for the prominent lymphoplasmacytic infiltrate. Though axillary lymph node involvement is common, only low axillary group of lymph nodes will be usually involved. The prognosis will be better than IDC, NOS type[10, 18].

**5. ATYPICAL MEDULLARY CARCINOMA**:

The tumor shows following characteristic features,

a. Syncytial growth comprising > 75% of the tumor
b. Atypical features
c. Focal tumor infiltration at the margins
d. Uniform nuclei and rare mitosis
e. Mild to absent lymphoplasmacytic infiltration at the margins.
f. Focal tubule formation[23].

**6. INVASIVE PAPILLARY CARCINOMA**

The tumor is rare and occurs more frequently in the postmenopausal women. Papillary carcinoma commonly present as in situ lesions. The invasive component can be papillary or it may show features of IDC, NOS type. As the presence of invasion in these tumors is not clear-cut, it should be applied only for cases with well differentiated true papillary structures. When a tumor with

papillary pattern is seen, metastatic papillary carcinoma from other sites should also be excluded[10, 18].

The tumor may have axillary lymph node metastasis particularly in solid variant of papillary carcinoma. Prognosis of the tumor is better compared to that of invasive ductal carcinoma, NOS type[24].

## 7. INVASIVE MICROPAPILLARY CARCINOMA

Invasive micro papillary carcinoma is a distinct rare variant of invasive ductal carcinoma. When the micro papillary pattern is found throughout the tumor it is referred as pure invasive micro papillary carcinoma. When it is present as a part of conventional IDC it is called as mixed invasive micro papillary carcinoma**.** But the criteria to distinguish these two are not clear cut. Some authors suggest at least 50 % of the tumor should be micro papillary to call it as pure invasive micro papillary carcinoma.

### MICROSCOPY

The tumor is composed of clusters of cells arranged in micro papillary or tubular pattern and tumor cells are found floating in clear spaces. Fibro vascular core will be absent in the micro papillary clusters. The clusters exhibit a “inside out” arrangement in which the apical cells are polarized outside and this can be evidenced by MUC 1 staining.

The nuclear grade of this tumor cells will be high. About half of the cases may show psammoma bodies. In situ component seen in these cases is usually micro papillary and sometimes cribriform pattern**.**

Lymphatic invasion is reported in more than 50 % of the cases. The tumor has a bad prognosis.

**IHC:**

In a study done by Cruz et al, estrogen receptor was positive in 72-75 % of cases, 45 % cases were positive for progesterone receptor and 36 % of the cases show Her 2-neu overexpression[25].

## 8. APOCRINE CARCINOMA

Apocrine carcinoma is very rare comprising 0.5% of all breast carcinomas. The tumor is composed entirely or predominantly of apocrine type cells with tumor cells being large with abundant eosinophilic cytoplasm with vesicular nucleus and prominent nucleolus. Glandular differentiation can be seen with apocrine snouts. Diagnosis of apocrine carcinoma should only be made when the architectural features are those of a malignant tumor. Immunohistochemically they are positive for GCDFP-15.Estrogen and progesterone receptors will be negative[26].

## 9. SECRETORY CARCINOMA

Secretory carcinoma is a rare tumor and usually seen in children. It can also occur in adults and has an excellent Prognosis.

**GROSS:**

The tumors are usually small and well circumscribed.

**MICROSCOPY:**

The tumor is composed of tubuloalveolar and papillary structures. The lumina contain eosinophilic PAS positive, diastase resistant material. The malignant cells have a pale staining vacuolated cytoplasm. Nucleoli may be prominent and mitosis is scanty.

**IHC:**

There is a strong immunoreactivity for S-100 and a-lactalbumin.

**10. METAPLASTIC CARCINOMA**

Metaplastic carcinoma represents tumor predominantly with cell type other than epithelial and glandular component. It includes many categories which may overlap with each other. Metaplastic carcinoma is more aggressive than invasive ductal carcinoma. Metastasis is usually hematogenous rather than lymph node metastasis.

**GROSS:**

The tumors are circumscribed and firm to hard in consistency. Degenerated cystic areas can be seen in cases with squamous metaplasia. Some of the tumors may have infiltrative borders.

## CLASSIFICATION OF METAPLASTIC CARCINOMA

### TABLE 1: CLASSIFICATION OF METAPLASTIC CARCINOMA

| Purely epithelial | Mixed epithelial and mesenchymal |
|---|---|
| • Squamous - Large cell keratinizing, spindle cell, acantholytic<br>• Adenocarcinoma with spindle cell differentiation<br>• Adenosquamous, including mucoepidermoid | • Carcinoma with chondroid metaplasia<br>• Carcinoma with osseous metaplasia<br>• Carcinosarcoma |

## A. SQUAMOUS CELL CARCINOMA

### GROSS

The tumors are large showing cystic spaces filled with keratin.

### MICROSCOPY:

In pure squamous cell carcinoma the central cystic cavity is lined by malignant squamous cells. Most cases represent squamous metaplasia.

Other two variants which can be seen are acantholytic squamous cell carcinoma and adenosquamous carcinoma. Low grade adenosquamous

carcinoma is said to have a favourable prognosis whereas acantholytic squamous cell carcinoma has an aggressive behavior[27].

**B. CARCINOSARCOMA**

When the transition between sarcomatous and carcinomatous component is gradual and sharp, it is termed carcinosarcoma. Microscopically the sarcoma like component can be malignant fibrous histiocytoma, osteosarcoma, chondrosarcoma, angiosarcoma or a combination of various components.

When the transition to cartilaginous or osseous elements is direct without an intervening spindle cell component or osteoclastic giant cells, it is called matrix producing carcinoma.

**IHC:**

The sarcoma like elements acquires vimentin and other mesenchymal features which is referred to as the phenotypic switch. The cells are occasionally positive for epithelial markers.

**SPREAD RELATED VARIANTS**

**1. INFLAMMATORY CARCINOMA**

The diagnosis of inflammatory carcinoma is essentially based on clinical criteria. Clinically the entire breast is red, warm mimicking mastitis. The mastitis like appearance is due to carcinomatosis of dermal lymphatic vessels. Skin biopsy is usually performed to reveal dermal lymphatic permeation.

Histopathological examination of some of the cases shows an undifferentiated carcinoma.

The prognosis is usually bad. Studies done by Charafe-Jauffret et al. found that most of the inflammatory carcinomas are negative for estrogen and positive for MIB1, E-Cadherin and HER2/ neu[28].

**INVASIVE LOBULAR CARCINOMA:**

**1. CLASSIC TYPE**

It is the most typical form of Invasive Lobular Carcinoma which is characterized by the presence of small, uniform tumor cells which grows singly, in Indian file and in a concentric fashion around lobules. The stroma is usually abundant, of dense fibrous type[10, 18].

**2. PLEOMORPHIC LOBULAR CARCINOMA**

This form of invasive breast tumor has the pattern of growth of a classic breast carcinoma but exhibits a marked degree of nuclear pleomorphism and abundant cytoplasm. It also frequently shows apocrine differentiation, focal signet ring morphology.

**IHC:**

These tumors lack hormone receptors with higher expression of P53 and HER2/neu. They occasionally express chromogranin, but lack E-cadherin staining[10].

## 3. HISTIOCYTOID CARCINOMA

Histiocytoid carcinoma is characterized by a diffuse growth of tumor cells which displays abundant granular, foamy cytoplasm. It may simulate the appearance of a granular cell tumor (myoblastoma). It is currently viewed as a variant of invasive lobular carcinoma exhibiting apocrine differentiation.

**IHC:**

Immunohistochemical reactivity for GCDFP-15 and the demonstration of mRNA for the related prolactin-inducible protein (PIP) by in situ hybridization. In most cases E-cadherin is absent. The mucins expressed by this tumor include some 'non-mammary' types, such as MUC2 and MUC5AC[10].

## 4. SIGNET RING CARCINOMA

Signet ring carcinoma is a type of breast carcinoma in which the tumor cells show intracytoplasmic mucin accumulation, resulting in the typical signet ring appearance. It is important to separate this tumor from mucinous carcinoma (in which the mucin is extracellular).

**IHC:**

Signet ring carcinoma is positive for CK7 and MUC1, and usually negative for E-cadherin.

## 5. TUBULOLOBULAR CARCINOMA

This variant is characterized by the admixture of small tubular formations having a minute or undetectable lumen ('closed' or 'almost closed' tubules) with

cords of tumor cells growing in a lobular configuration similar to that of invasive lobular carcinoma. The in situ component, if present, may be of lobular, ductal or mixed type. It is associated with a higher incidence of multifocality and positive axillary nodes than pure tubular carcinoma

**IHC:**

Immunohistochemical profile is intermediate between those of ductal and lobular carcinoma, in that it shows positivity for both E-cadherin and HMW keratin[10, 20].

## HISTOLOGICAL GRADING OF DUCTAL CARCINOMA

Grading of breast cancer was first attempted by Greenhough in 1925[29]. It was an extensive system with 18 features and it is not used now. In 1993 Haagensen evaluated 15 histological features which mainly include growth pattern, cell morphology and the stromal reaction.

The most popular grading system till date was proposed by Bloom in 1950[30]. The original classification proposed by him was based on three main features which include degree of tubule formation, nuclear features and mitotic activity. He classified breast carcinomas into 2 group low grade and high grade tumors.

In 1957 this classification was upgraded by modifications of Bloom and Richardson .without changing the features of classification, he included score of 1 to 3 to each criteria according to mild ,moderate or marked degrees. A total score of 3 to 9 was given as follows[31],

**TABLE 2: BLOOM AND RICHARDSON GRADING SYSTEM 1957**

| | | |
|---|---|---|
| Score 3-5 | Grade 1 | Well differentiated tumors |
| Score 6-7 | Grade 2 | Moderately differentiated tumors |
| Score 8-9 | Grade 3 | Poorly differentiated tumors |

Elston further modified this classification by applying this only to invasive ductal carcinoma and excluding special types like mucinous, medullary carcinoma. Elston and Ellis modified Bloom and Richardson grading system by quantifying the mitotic activity[32]. This is also referred as the Nottingham modification of Bloom and Richardson system.

**TABLE 3: NOTTINGHAM MODIFICATION OF BLOOM RICHARDSON HISTOLOGICAL GRADING SYSTEM**

| CRITERIA | SCORE |
|---|---|
| **Tubule and gland formation** | |
| Majority of tumor (>75%) | 1 |
| Moderate degree (10-75%) | 2 |
| Little or none (< 10% ) | 3 |

| **Nuclear pleomorphism** | |
|---|---|
| Small, regular, uniform | 1 |
| Moderate variation in size, shape | 2 |
| Marked variation in size, shape | 3 |

**Mitotic count**

Mitotic count is also graded as 1-3. But it depends on the field diameter used. Mitotic figures are to be counted from the most mitotically active area. 10 high power fields should be counted from the same area. Poorly preserved area should be ignored.

**TABLE 4: SCORING OF MITOTIC COUNT**

| Field diameter 0.59mm | Field diameter 0.44mm | Score |
|---|---|---|
| 0-9 | 0-5 | 1 |
| 10-19 | 6-10 | 2 |
| >20 | >11 | 3 |

**TABLE 5: FINAL GRADE OF NOTTINGHAM MODIFICATION OF BLOOM RICHARDSON HISTOLOGICAL GRADING SYSTEM**

| GRADE | SUM OF POINTS |
|---|---|
| I | 3–5 |
| II | 6–7 |
| III | 8–9 |

## PROGNOSTIC FACTORS

### 1. Age

Prognosis is better in patients <50 years. Older patients have a higher rate of recurrence and distant metastasis.

### 2. Pregnancy

Carcinoma breast manifesting during pregnancy is generally aggressive with over expression of HER2/neu and low expression of hormone receptor.

### 3. BRCA-1 status

Tumors associated with BRCA1 mutation carriers have a low survival rate.

**4. Skin and nipple invasion**

T4a lesions are associated with decreased survival rate. Nipple involvement is associated with high incidence of axillary node metastasis[33].

**5. Presence or absence of invasion**

The single most important prognostic determinator of breast carcinoma is the presence of invasive component. The invasive component of a tumor correlates with the nodal metastasis. The in situ component is proportionate to the incidence of multicentricity and indirectly with occult metastasis[10].

**6. Size of the tumor**

Tumor diameter should be measured in three planes to the nearest millimeter. The greatest diameter is taken as the size of the tumor. For lesions less than 1 cm stage micrometer is used in histological sections for tumor size estimation. The invasive component is the better predictor of the total tumor size than the DCIS component.

It has been proved beyond doubt that tumor size correlates with the prognosis. Multivariate analysis by Nottingham/Tenovas Primary Breast cancer study showed tumor size is an independent prognostic variable[34].

**7. Histological type**

The variants of invasive ductal carcinoma with a better prognosis than IDC, NOS type are tubular carcinoma, cribriform carcinoma, medullary carcinoma, papillary carcinoma, pure mucinous carcinoma and secretory

carcinoma. Signet ring cell carcinoma in particular is associated with a poor prognosis.

**8. Histological grade**

The poorer the grade of the tumor by Nottingham modification of the Bloom–Richardson system, the worse is the outcome, particularly IDC, NOS type[35].

**9. Tumor necrosis**

Spontaneous tumor necrosis is associated with tumors showing high histological grade and increased incidence of lymph node metastases[36] and hence a poorer prognosis.

**10. Lymphovascular emboli**

Presence of tumor emboli within endothelial lined spaces in the peritumoral area under H & E is taken as positive lymphovascular invasion. Lymphatic emboli within the breast correlate with local recurrence and vascular invasion correlates with distant spread[37]. Tumors with vascular emboli have a poor prognosis.

**11. Lymph node status**

Lymph node involvement is an important prognostic factor[38]. The better method for lymph node assessment is by histopathology than clinical assessment. Many studies showed that patients with regional node involvement have a bad prognosis than those without node involvement.

Ten year survival rate for node negative patients will be 75 % while compared to that only 25-30% in node positive patients. Prognosis is also dependent on the number and the level of regional lymph nodes. The prognosis will be poor if greater number of nodes is involved[39].

NSABR categorises patients under two divisions for therapeutic purpose. They are categorized as patients with 1-3 positive nodes and cases with 4 or more positive nodes.

**12. Hormone receptors**

Estrogen receptor positive tumors have a longer disease free survival.

**13. HER2 /neu expression**

HER2 / neu over expression correlate with the tumor grade. It has a poor prognosis especially when associated with lymph node metastasis. It is an excellent predictor of response to the drug trastuzumab but a weak predictor for chemotherapy[40].

**14. Cell proliferation**

Cell proliferation has emerged as an important parameter especially in node positive patients. Tumors with increased proliferation rate behave aggressively. The simple method to assess proliferation will be the mitotic count .Nowadays Ki 67 has been used to determine cell proliferation by immunohistochemistry[41]. Other methods include flow cytometry, S phase fraction.

**15. Microvessel density.**

Invasive breast carcinoma with prominent vascular component in the surrounding stroma behaves aggressively than other tumors[42].

**HORMONE RECEPTORS**

The presence of hormone receptors (estrogen, progesterone) correlates with the response of the tumor to endocrine therapy and chemotherapy. Though estrogen and progesterone are codependent variables, estrogen receptor status is the powerful predictive factor in breast cancer management[43].

ER and PR are expressed in 80 % and 60 % of the breast cancer, respectively. There is not much correlation between receptor positivity and cyto architectural type of breast. But however some studies suggest that mucinous, tubular, lobular carcinoma show high estrogen positivity. Medullary, apocrine, metaplastic carcinomas are estrogen negative[44].

There are two parameters evaluated in immunohistochemical assessment of hormone receptors which include,

- Number of tumor cell nuclei stained-expressed as percentage of entire tumor nuclei population.
- Intensity of the reaction

These hormone receptors are measured semiquantitatively. There are various scoring systems like Quick score, H score and Allred score with Allred score is the most established score these days. It consists of a score for intensity

(0 to 3) and a score for the proportion of nuclear staining (0 to 5). The final score is obtained by the sum of proportion score and intensity score which ranges from 0 to 8. Invasive tumor with an Allred score of more than 2 was considered to be positive for hormonal receptors[45].

**Estrogen and Progesterone receptors**

The ovarian hormones, primarily estrogen are believed to play a role in breast cancer etiology. These steroid hormones influence their effect on breast cell proliferation by the estrogen receptor (ER) and progesterone receptor (PR). The estrogen receptor is expressed in two different types, usually referred to as α and β. They are encoded by gene ESR1 and ESR2 on the sixth and fourteenth chromosome (6q25.1 and 14q), respectively[46]. The main function of the estrogen receptor is as a DNA binding transcription factor, which regulates gene expression[47].

The binding of estrogen to ER stimulates proliferation of mammary cells, resulting in increase in cell division and DNA replication leading to mutations. Secondly, estrogen metabolism produces genotoxic waste. Both processes lead to disruption of cell cycle, apoptosis and DNA repair, progressing to tumor formation. This is the proposed mechanism of effects of estrogen on breast cancer. ER-α is certainly associated with more differentiated tumors, while evidence of ER-β involvement is controversial[48].

The source of estrogens is not only endogenous but the use of exogenous estrogen, such as oral contraceptives and hormone replacement therapies (HRT),

also triggers this cell proliferation process. Hunter et al. have studied this effect of oral contraceptives as sources of exogenous estrogen for the increased risk of breast cancer for women using these products[49]. Estrogen receptor status is an important diagnostic parameter when a patient presents with breast cancer. Patients are classified as either estrogen receptor positive (ER+) or estrogen receptor negative (ER-) based on immunohistochemistry of the biopsy sample for ER-α.

The introduction of adjuvant hormonal therapy, in particular tamoxifen, has been one of the major breakthroughs in the fight against breast cancer mortality.

For women with estrogen receptor (ER)-positive breast cancer, 5 years of tamoxifen in an adjuvant setting decreases the risk of death. The same effect cannot be said for ER-negative patients as risk of death from breast cancer was significantly increased in those treated with tamoxifen[50].

Progesterone an ovarian steroid hormone plays a key role in the development and functioning of the mammary gland. As with other steroid hormones, the action of progesterone is mediated through its intercellular receptor, the progesterone receptors (PR) which function as a transcription factor that regulates gene expression. The tumorigenic effect of progesterone is mediated by mutation or aberrant expression of the coregulators which affect the normal function of the PR and may lead to breast cancer[51].

The prognostic significance of elevated PR levels is that these tumors are less aggressive tumors. The other good prognostic feature is that they are associated with a longer overall survival time in metastatic disease, whereas PR negative tumors are more aggressive[52]. The American Society of Clinical Oncology (ASCO) recommended that ERα and PR should be measured on every primary invasive breast cancer.

**Human Epidermal growth factor Receptor 2 (HER-2/neu)**

HER-2 (Human Epidermal growth factor Receptor 2) also known as proto-oncogene Neu .The oncogene neu is so-named because it was derived from a rodent glioblastoma cell line, which is a type of neural tumor. HER-2 protein is known to form clusters in cell membranes that might play a role in tumorigenesis[53]. HER cellular signalling occurs through transmembrane receptor tyrosine kinases and can induce cell proliferation, motility, and invasion. Dysregulated expression and activity of HER family members is prevalent in human neoplasia. Overexpression of this protein leads to constitutively activated tyrosine kinase, resulting in mitogenic transduction and poorer prognosis[54].

Approximately 10% to 34% of invasive breast cancers overexpress the HER2 receptor and are referred to as HER2-positive HER2+[55]. Overexpression is associated with poor prognosis as well as with aggressive tumor growth and metastases.

It also occurs in other cancers such as ovarian cancer, stomach cancer, and uterine cancer[56]. The American Society of Clinical Oncology (ASCO) recommended that should be determined Her-2 as a part of diagnostic routine on every primary invasive breast cancer.

Studies show that HER2/neu is an independent prognostic indicator for overall survival of patients with breast carcinoma.

**IMMUNOHISTOCHEMISTRY**

Immunohistochemistry is one of the powerful ancillary methods used in pathology today which has revolutionized the study of disease and its prognosis. The most useful aspect of IHC is that it is a powerful and cost effective tool applicable in light microscopy. The morphologic observations made by pathologists are validated by the use of IHC. Immunohistochemistry (IHC), or immunocytochemistry, is a method for localizing specific antigens in tissues or cells based on antigen-antibody recognition[57].

The main advantages of IHC which makes it a good companion to pathologist are, it can be done in regular laboratories under light microscope without specialized devices. Standard fixation techniques can be used and it is permanent which can be done on archival material with additional benefit of good sensitivity and specificity[58].

**USES OF IHC:**

- Classifying undifferentiated tumors, lymphomas, neuroendocrine and soft tissue tumors.
- Detection and accurate assay of tumor biologic factors of prognostic and predictive values such as hormone receptors (ER, PR) and HER-2/neu in breast cancer.
- Detection of metastatic cells in bone marrow, lymph nodes& serous fluids when the cell groups are too less or confusing.

Clearly the validity of immunohistochemistry in diagnostic histopathology depends in great measure on the quality of immunostains. In addition to antibody quality, three other factors have a major impact on immunohistochemistry. 1. Tissue fixation and processing. 2. Unmasking of epitopes. 3. Sensitivity of detection system. Among the various available fixatives, formaldehyde is the most popular because of its low cost, ease of preparation, and because it preserves morphologic details with few artifacts. However, formaldehyde fixation results in a variably reversible loss of immunoreactivity by its masking or damaging some antibody binding sites.

IHC is performed in formalin fixed paraffin embedded tissue blocks. The results were interpreted based on Allred scoring system for ER, PR and by using the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines for HER 2[(59)].

**TABLE 6: ER, PR SCORING BY IMMUNOHISTOCHEMISTRY**

**(ALLRED SCORING SYSTEM)**

| **% of cells positive** | **score** | **Staining intensity** | **Score** |
|---|---|---|---|
| 0 | 0 | - | - |
| < 1 | 1 | Mild | 1 |
| 1 – 10 | 2 | Moderate | 2 |
| 11 – 33 | 3 | Intense | 3 |
| 34 – 66 | 4 | | |
| 67 – 100 | 5 | | |

TOTAL SCORE =PROPORTION SCORE + INTENSITY SCORE ( 0 TO 8)

**INTERPRETATION:**

0, 2 - Negative

≥ 3 - Positive

**TABLE 7: GRADING OF THE IMMUNOHISTOCHEMICAL STAINING FOR HER 2 / NEU OVEREXPRESSION**

| Score | Staining Pattern | HER 2 / neu protein overexpression assessment |
|---|---|---|
| **0** | No staining at all or very slight partial membrane staining in less than 10% of tumor cells. | Negative |
| **1+** | Faint barely perceptible membrane staining in more than 10% of tumor cells. Cells stained in only part of the membrane. | Negative |
| **2+** | Weak to moderate complete membrane staining observed in more than 10% of tumor cells. | Weakly Positive |
| **3+** | Strong complete membrane staining in more than 30% of tumor cells | Strongly Positive |

## MOLECULAR SUBTYPE OF BREAST CARCINOMA

In the year 2000 Perou and colleagues tried to segregate breast carcinoma based on their gene expression profiles into distinct subgroups. They used microarray to demonstrate gene expression. It was accepted with a hope that this will provide new approach to the biology of breast cancer and was also believed that it may impact on the therapeutic approach of the patient[60].

The subtypes recognized by the gene expression are luminal A, luminal B, HER2/neu type, basal like and normal breast like. It was suggested that normal breast like subtype is most propably an artifact rather than genuine subtype. It is due to lack or sparsity of tumor in the tissue samples used for microarray analysis.

As the different subtypes show specific characteristics, they may benefit from different therapeutic approach. Among the subtypes, basal like type shows the worst prognosis[60].

## LUMINAL A

It comprises 50% of the invasive breast cancer. This is seen in tubular carcinoma, cribriformcarcinoma, low grade invasive ductal carcinoma NOS type and classic lobular carcinoma. It expresses luminal cytokeratins and hormone receptors. They respond well to chemotherapy and have good prognosis.

**LUMINAL B**

It comprises 20% of invasive breast carcinoma. It corresponds to invasive ductal carcinoma NOS type and micropapillary carcinoma. It expresses cytokeratins with moderate to weak expression of hormone receptors. Proliferation rate is higher compared to that of luminal A type. It shows variable expression for chemo and endocrine therapy.

**HER 2/NEU**

It comprises about 15% of invasive breast cancer. The tumors are usually high grade with lymph node metastasis. They are negative for hormone receptors. HER2/neu amplification and high proliferation is seen. Patients respond to trastuzumab and anthracycline based chemotherapy and usually have a poor prognosis.

**BASAL LIKE**

It comprises about 15% of invasive breast carcinoma. High grade IDC NOS, metaplastic and medullary carcinoma come under this category. They are negative for hormone receptors and HER2/neu but show a high proliferation rate.

An effort is also made to classify the tumors based on the immunnohistochemical expression. The panel of markers include estrogen receptor (ER), progesterone receptor, HER2/neu, EGFR, cytokeratin 5/6 and Ki67.

Based on the reactivity they are classified as follows,

**TABLE 8: MOLECULAR TYPING OF BREAST CARCINOMA ACCORDING TO EXPRESSION OF IHC MARKERS**

| **Immuno profile** | **Luminal A** | **Luminal B** | **HER2/neu** | **Basal-like** |
|---|---|---|---|---|
| ER, PR | ER and/or PR+ | ER and/or PR+ | ER–, PR– | ER–, PR– |
| HER2 and others | HER2 – Low Ki-67 (<14%) | HER2+ or HER2 – Ki-67 =14% | HER2+ | HER2– CK5/6 and/or EGFR+ |

But this classification has some drawbacks. Relatively small number of cases are used do define molecular subtypes and few less common distinct subtypes are missed out. The basal like subtype also contains tumors with favourable prognosis like medullary carcinoma, secretory carcinoma which show the need of a low grade basal like subtype. Currently the clinical value of molecular classification is not well established[61].

## TISSUE MICROARRAYS

Dr. Hector Battifora introduced a new technique in 1986 in which a number of tissues from various organs are thrown into the same block and tissue distribution of a particular antigen was processed. This was called sausage block technique[62]. Wan et al in a modification of the original technique produced a library of paraffin embedded cores and used it to determine various staining patterns of many number of monoclonal antibodies[63]. Later this was modified by Kononen et al who introduced the term 'Tissue microarray' in 1998 which is widely used nowadays, which includes the usage of 4mm skin biopsy punch needle[64].

The major focus of TMAs at the present time is in the fields of cancer and non-cancer research; and quality control in modern pathology[65]. It has greatly facilitated the in situ analysis of molecular targets at the DNA, mRNA, and protein levels under standardized conditions in a large number of archived pathology specimens[66].

One of the major advances of the technique is the ability to perform a variety of studies like immunohistochemistry, fluorescence in situ hybridization (FISH), or RNA in situ hybridization (ISH) in a very cost effective manner[67]. The common application of microarray technique is for immune staining markers such as p53, bcl2, HER2, E-cadherin, ER, PR, Ki67 and others[68]. Despite the flexibility and benefits of this method, its broader use has been hampered because of the technical difficulties of array construction and the costs

of available arrayers. The specialized commercial automated and semi-automated equipments as well as the inexpensive simple manual tissue array methods have been used to construct the TMA blocks[69]. The cost of available arrayers and technical difficulties of routine array construction have limited the development of tissue microarray technique in developing countries like India[70].

The disadvantages associated with this technique are that the small size of TMA cores may not be representative of the whole heterogeneous cancers and may not provide enough data about the entire tissue profile[71]. The technical issues commonly noted with the application of microarray technique are sectioning difficulties, irregular cutting plane due to variable thickness of donor paraffin blocks, core losses and instability of the TMA blocks in routine TMA.

This technology thus offers a way to fill the gap between the discovery of new molecular markers, derived from high throughput genomic analysis, and their application in the clinical setting.

**Materials for TMA**

The sources of the material for construction of Tissue microarray varies widely and are categorized based on material of origin. They are called simply as Tissue microarrays are constructed from paraffin embedded materials and can also be constructed using resin as recipient block, if very thin sections are required[72]. But the construction of resin Tissue microarray is very difficult when compared to paraffin Tissue microarray[73]. Tissue microarrays can also be

constructed using frozen sections in which case they are called as Cryoarrays or and can be constructed using paraffin embedded cell lines and by using cell blocks[74].

**TMA categorization**

Tissue microarray is categorised based upon the usage of instruments and purpose of tissue microarray. Based upon the usage of the instruments and microarrayer used it has been classified into manual, semi-automated and automated tissue microarray. There are many instruments which are available nowadays which includes, the manual and automated tissue arrayer from Beecher instruments, the semi-automated tissue arrayer from Veridiam, the quick ray manual and the automated tissue arrayer from Unitma and the manual array mold tissue arrayer. Apart from this many number of home- made tissue arraying methods have been published[75, 76].

The common feature between all these devices is that they use hollow needles or punches and they adopt the technique of skin biopsy to extract tissue cores from a donor block and make a new paraffin recipient block. After cutting all the tissue cores appear as circular samples arranged in grid like fashion.

The initial step which is considered most important in construction of Tissue microarray is to clearly define the purpose of Tissue microarray construction and to decide the number and size of the tissue core which should be taken from the donor block. Then blocks and slides of the selected cases are carefully reviewed to mark the area of interest. The method of tissue sampling

varies widely from tissue to tissue. If task is to compare the expression pattern of a marker from tumor centre and periphery, the cores from the particular location may be compatible. However the method of tissue sampling is entirely different when the task is to characterize the overall expression of the protein in a tumor. Targeted sampling technique is followed in the cases of comparing the expression patterns of tumor centre and periphery whereas Random sampling technique is best suited to study the overall expression patterns[77].

**Fig. 2(A) shows taking out a tissue core from the donor block with skin biopsy needle.**

Tumor heterogeneity, which results in differential expression of different tumor cell, has been recognized as the potential problem in tissue sampling for the construction of Tissue microarray. Taking multiple samples from each tumor or area of interest appears to be the best technique to overcome this problem[78].

Although no standard and universally agreed sampling methods are in the records, it is intuitive when more samples are taken it becomes the representative area for donor tissue. The concordance of Tissue microarray technique with full section eventually depends upon the number of cores obtained. Most of the studies seem to indicate that the results from triplicate Tissue microarray cores have upto 98% concordance with the results from full section. However Goethal through his study suggests that atleast four cores are needed to achieve greater accuracy of more than 95% which is obtained using two core tissue sampling method[79].

It should be also noted that there are also technical reasons which increases number of cores taken from each tissue block[80]. The reasons being, tissue folding and complete loss of tissues during processing and section cutting. Total number of lost cases accounts for as high as 23% in the Tissue microarray construction study by using tissue cores from the cases of renal cell carcinoma[81]. There are controversial data regarding the size of the cores that should be used in Tissue microarray technique and their influence over the technique.

The next critical step included in tissue microarray is designing the layout of the tissue microarray. There are no general agreements regarding the designing of optimal layout of a tissue microarray. As there are problems of staining artefacts when performing immunohistochemistry in the full sections, the use of protection wall in the Tissue array technique is recommended as

introduced by Hoose et al, which uses a row of tissue cores which will not be analysed and can be any tissue that is available in plenty in the laboratory[82]. Any confusion in identifying the origin of the cores after Tissue microarray construction makes the staining and analysis very difficult and hence orientation of the tissue cores needs to be perfect. Many use orientation cores in the specific position, usually outside the geometric margin of the array. Using the intentionally left empty core position, it is possible to identify and orient the position of the cores macroscopically as well as to orient cut section microscopically. In addition insertion of control tissue array may be of more value in orientation of the tissue cores. Thus the control core serves as an orientation control and also as both internal positive and negative control[83].

Recipient blocks are nothing but the empty paraffin blocks that are prepared by pouring the soft molten wax into existing metal moulds of varying size. The major difficulty encountered in using metal moulds is the formation of air bubbles within the recipient blocks during the cooling procedure. The air bubbles which are formed during the process of cooling will not be evident apparently and not be identified till the section cutting. The subsequent sectioning of Tissue microarray causes severe distortion of constructed array and leads to difficulties during the steps of interpretation and analysis. To minimize this kind of problem and as a quality control measure, all the recipient blocks are subjected to X-ray by using Faxitron machine before Tissue microarray construction and if air bubbles are found the recipient blocks are melted for reuse. The use of moulds which are made from paper and plastic may reduce the

problem of bubble formation which is commonly encountered when paraffin recipient blocks are used[83].

**Tissue Arrayers**

Both automated and manual tissue arrayers are available for the construction of Tissue microarray. Automated tissue arrayers are easy to use. The instrument usually marks, edits and saves punch co-ordinates by using an on screen display and software tools. Automated tissue arraying instruments are commonly used in laboratories with high volume of Tissue microarray and can punch upto 180 cores/hour.

**Automated HT-1 Tissue Microarray**

Automated Tissue microarray is a highly potential and efficient technique which can be used to study gene expression. At present TMA has become a standard research platform for the histopathological analysis. This technique involves the acquisition of multiple core biopsies from area of interest from the donor block and transferring it into the recipient paraffin blocks with the help of special instrument. This includes collection and selection of donor blocks to be analysed, identifying the area of interest and representative areas, preparation and making array pores in the recipient paraffin blocks, punching tissue cylinders from the area of interest from the donor block, insertion of cylinders containing tissues into the recipient blocks and embedding and cutting of newly formed Tissue microarray. However most important of all these steps is the construction of tissue microarray blocks using commercially available

instruments. The key steps in automated tissue microarray are punching array pores in recipient blocks and embedding multi-tissue cylinders into the recipient blocks[84].

**Recipient block formation**

This method uses three types of recipient block maker which are called as recipient block-molding machine that can accommodate 24, 42, 56 tissue cylinders. The spacing between the cores is usually fixed. Recipient paraffin blocks can be made within several seconds by using block-molding machine. The instrument is composed of an array pores forming metal tamp series of metal embedding boxes and punch needles with corresponding inner cores. The array pores-metal consist of metal plates and a bracket. The lower plate is fixed with hollow cores while upper plate is fixed with inner cores. The inner diameter is designed from 0.5 to 2.5mm.The instrument for making array recipient blocks is fixed in the manipulator with the help of the bracket. The lower plate with the hollow array can move up and down by controlling the handle. The residual paraffin in array hollow punch is automatically removed by the piston[84].

**Negative-pressure embedding**

Negative pressure embedding instrument is designed to re-embed the multi-tissue cylinders into the recipient block for the construction of Tissue microarray. It is composed of negative pressure room, vacuum pump and an embedding box. The embedding box is situated in the negative pressure room and is connected to the vacuum pump through the tube. The bottom of the

embedding box consists of metal mesh, which can effectively adjust the negative pressure so that the air bubbles which are formed can be drained out of recipient paraffin blocks. Screen mesh can also be used to adjust the temperature for the embedding media[84].

**TMA without prefabricated recipient block**

Array construction done with the help of automated tissue arrayers proved to be very costly and hence not suitable in developing laboratories. Hence efficient microarray system which is cost effective was designed using manual tissue arrayer technique. Most of the manual methods used pre constructed paraffin recipient block into which holes are punched followed by insertion of tissue cores. The use of paraffin blocks proved difficult because of block breakage during punching, non alignment of holes, and mismatch between the size of the recipient hole and the tissue core. With the desire to overcome these difficulties, TMA construction was done without the use of prefabricated paraffin blocks[85].

This technique followed the method which was initially modified by Kononen et al. A disposable skin punch biopsy needle of 2mm diameter was used for punching cores from the donor blocks. A long steel wire with a blunt end was modified into a stylet. The skin biopsy needle has the sharp cutting edge and a uniform cylindrical core. Double sided adhesive tapes, pair of stainless steel forceps, paper cutters, embedding mould and plastic cassettes are the additional requirements of this technique. A piece of double sided adhesive tape

is cut according to the size of embedding mould and the top surface is exposed to receive the tissue cores. The site for the attachment is marked with the ruler and the felt pen. The area to be cored is marked on the block by superimposing the marked area over the slides. After obtaining the core from the donor block, the core is transferred to adhesive tape using the forceps. Then it is transferred to stainless steel mould and after which melted paraffin wax is poured. Later after uniform setting of the block the adhesive tape is peeled off to expose the cutting surface. This method can give rise upto 20 sections from each core. This is reliable, readily reproducible and does not need any specialisation[85].

**Simple manual Tissue Microarray**

This is the modification of conventional manual tissue microarray which used skin punch biopsy needle. This technique used bone marrow aspiration needle for the construction of manual tissue microarray. Both 14 gauge and 16 gauge needles are used. After the selection of donor block, the area of interest is marked and empty recipient paraffin block is made using the mould. By using 16gauge needle pores are made in the recipient block[85].

Then with the help of 14 gauge needle tissue cores are obtained from the area of interest from the donor block and then inserted into the recipient block. The smaller diameter (16G) of the needle used to punch the blank paraffin wax block allowed the bigger tissue cores (14G) to fit exactly into the blank. Once the array is complete hot liquid paraffin is poured over the array surface and the

tissue cylinders are levelled with the blocks using a glass slide. Then the array is incubated at $60^0$c for 15 minutes after which the array is chilled on ice [fig 2(B)].

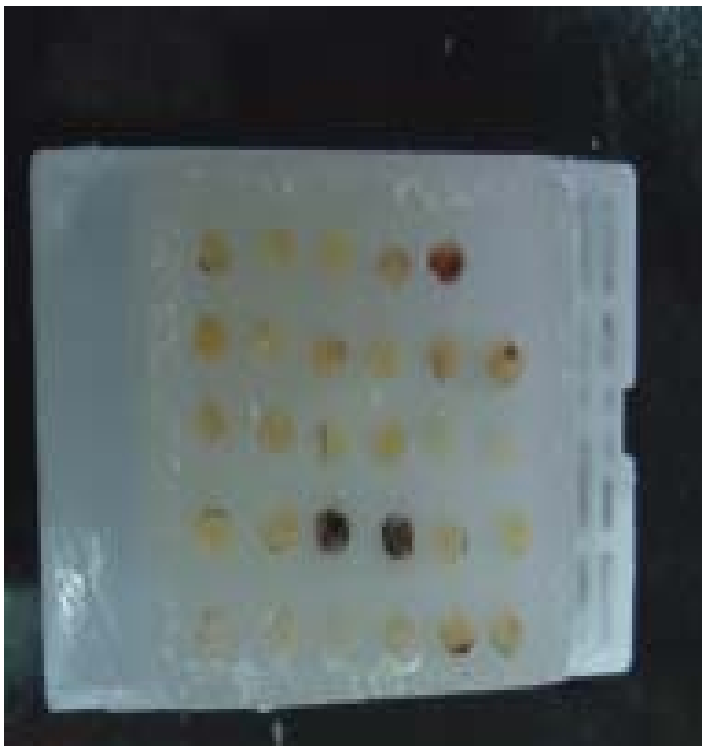


**Fig. 2(B) – shows the final recipient paraffin block**

**Types of TMA based on application**

The technique of tissue microarray can be classified based on the purpose of Tissue microarray construction. Random arrays contain tissue from multiple sites including both tumoral and non-tumoral tissues. This is most widely used for monitoring the efficacy of the existing antibodies. Cell line arrays consist of normal and cancer cell lines that are grown in cultures. The main purpose of using this array is to analyse the utility of an antibody in detecting the proteins. Outcome based arrays are the most valuable and the most difficult to construct

as they involve the collation of tissues from the patients of same disease, those who were exposed to similar pattern of treatment and have been followed up for significant period. Progression arrays are used in analysis of role of proteins in the progression of cancer. Tumor characteristic based array is constructed based on the given characteristics such as patient age and tumor grade[83].

The use of tissue microarrays has its own advantages and disadvantages. Tissue microarray allows the performance of tissue based array such as immunohistochemical analysis, histochemistry and in situ hybridisation on a very large number of sample in a cost effective manner [fig 2(C)]. Several different tissues from a number of patients can be examined in short period using TMA. Automated Tissue microarrayers are very quick and manual tissue arrays are simple to construct and cost effective. The major disadvantage of using Tissue microarray is that each tissue core obtained from the selected donor block represents only a fraction of the lesion. But this can be overcome by taking multiple cores usually from various sites[83]. Hence Tissue microarray can be considered as one of the superior and more advantageous technique used in studying tissue biology.

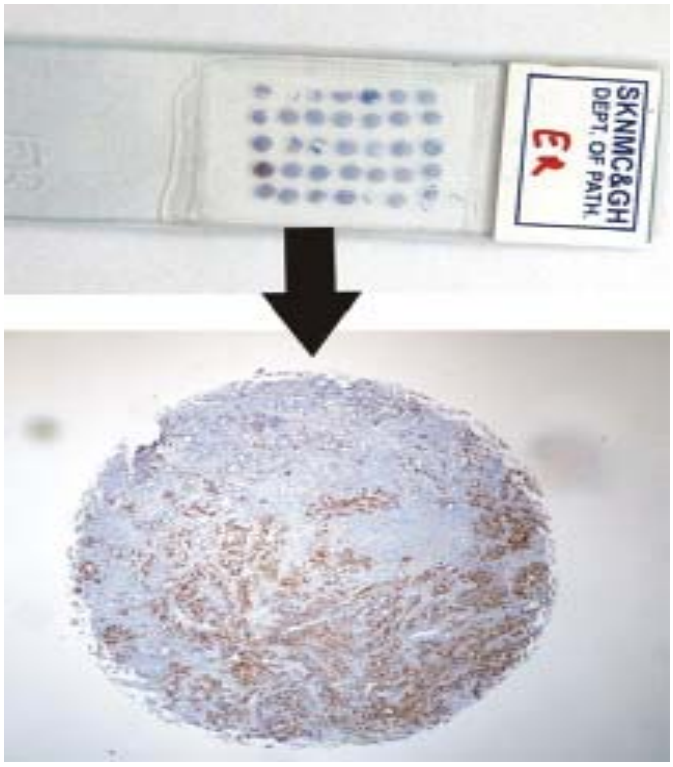



**Fig. 2(C) – Tissue Array slide stained with Immunostain for estrogen receptor (ER)**

# MATERIALS AND METHODS

The study was conducted after obtaining approval from Institutional Ethical Committee of Tirunelveli Medical College, Tirunelveli. The study was carried out in the Department of Pathology, Tirunelveli Medical College and Hospital, Tirunelveli from January 2014 to September 2015.

## SOURCE

Formalin fixed, paraffin embedded tissue blocks from 50 surgically resected breast tissues which were diagnosed as invasive breast carcinoma by histopathological examination were retrieved along with their haematoxylin and eosin stained slides and they were examined and the tumor was graded according to Modified Bloom and Richardson grading system.

## SAMPLE SIZE

A total of 50 cases were included in this study. Clinical data like patient age, sex and other relevant history were noted from the Pathology records.

## INCLUSION CRITERIA

- Invasive ductal carcinoma - NOS type
- Papillary carcinoma
- Medullary carcinoma
- Mucinous carcinoma
- Tubular carcinoma

- Metaplastic carcinoma
- Invasive lobular carcinoma

**EXCLUSION CRITERIA**

- Benign tumors of breast.
- Ductal carcinoma in situ.
- Lobular carcinoma in situ

**MATERIALS REQUIRED**

1. Lay out for constructing Tissue microarray.
2. Metal moulds and molten wax for preparing empty recipient paraffin block.
3. Donor blocks which contain formalin fixed paraffin embedded tissue obtained from all the cases of Invasive breast carcinoma.
4. Hematoxylin and eosin stained tissue sections made from the donor blocks.
5. Black glass marking pen for marking area of interest.
6. 16 gauge bone marrow aspiration needle for making punches in the recipient block and 14 gauge needle for obtaining core from the donor block.
7. Microtome and incubator for obtaining tissue sections and to dewax the sections
8. Positively charged slides for holding tissue sections for IHC.
9. Chemicals for preparing antigen retrieval solutions and for wash buffers.

10. Pressure cooker for antigen retrieval.
11. Kit for performing immunohistochemistry which includes primary antibody (ER, PR & HER 2 neu) and universal kit. Microscope, used for interpretation and grading of IHC.

**METHODOLOGY**

The method of performing immunohistochemistry over the paraffin tissue microarray includes the following steps.

1. Designing the layout for TMA construction.

2. Collection of the donor blocks.

3. Preparation of the recipient paraffin blocks.

4. Immunohistochemistry and analysis.

**DESIGNING THE LAY OUT**

Before constructing the array proper, the layout of the tissue microarray defining the geometric position of each tissue core in the recipient block is made. The grid is constructed in such a way that there was a single core from each case on the recipient block. The grid had blank cores in between the cores from the cases which helped in determining the position of the cases on the immunohistochemistry performed slides.

## COLLECTION OF THE DONOR BLOCKS

The hematoxylin and eosin stained sections which were prepared from formalin fixed paraffin embedded blocks of all the cases of invasive breast carcinoma in the Department of pathology during the study period were retrieved. The corresponding formalin fixed paraffin embedded tissues were also obtained which constituted the donor block. Then the hematoxylin and eosin stained slides which contained full sections were examined and the area of interest was marked by using black glass marking pen. The area of interest is the area of tumor containing well preserved and well stained malignant cells. Then these marked areas on the slides were matched with the donor blocks and the corresponding areas over the donor blocks were also marked with the help of black glass marking pen. This area was used as the site for obtaining cores for the recipient block.

## PREPARATION OF THE RECIPIENT PARAFFIN BLOCKS

The empty paraffin recipient blocks with minimum size of 25mm x 25mm were first prepared by freshly poured molten wax in the metal moulds. Then it was allowed to cool. Later using 16 gauge needle, paraffin wax cylinders of 2mm diameter were punched from the recipient blocks. Each block contained 3x3 cylinder matrix at a distance less than 2mm. In our study seven such blocks containing 50 cases were prepared.

Then using 14gauge bone marrow aspiration needle, tissue cylinders were obtained from the area of interest which were previously marked over the the

donor blocks, after which it was injected into the recipient blocks into the corresponding empty cylinders with the help of predesigned layouts so that six recipient blocks contained seven cases and one recipient block contains eight cases. After the recipient block was embedded with the tissue cores, the block was incubated at $40^0$c for 15 minutes and then it was allowed to cool for few minutes at room temperature and then the array was chilled on the ice for few minutes.

**IMMUNOHISTOCHEMISTRY**

**SECTION CUTTING**

Sections were taken at 5 microns thickness after tissue microarray construction on the surface of the Poly-L-Lysine coated slides. This was followed by incubation of slides at 58-$60^0$c for one hour.

**ANTIGEN RETRIEVAL SOLUTION**

We used two antigen retrieval solution and a wash buffer as prescribed by the manufacturer (PATH IN SITU).

1. Citrate buffer at a pH of 6.2 for HER 2/ neu.
2. Tris EDTA at a pH of 9 for ER, PR.
3. Tris wash buffer at pH of 7.6 for both.

## ANTIGEN RETRIEVAL

In our institution we followed antigen retrieval by using pressure cooker as it produces even heating with lesser disadvantages compared to other methods.

## PROCEDURE FOR IMMUNOHISTOCHEMISTRY AS GIVEN BY MANUFACTURER

1. Section cutting and incubation is followed by Xylene wash (2 changes) for 10 minutes each.
2. Rehydrated in graded alchohol containing 100%, 80%, 70% for ten minutes each.
3. Rinsed in distilled water for 2 minutes.
4. Antigen retrieval.
5. Cooling for 15 minutes.
6. Washed in TRIS wash buffer- 2 changes 5 minutes each.
7. Treated with peroxide block for 5 minutes.
8. Washed in TRIS wash buffer- 2 changes 10 minutes each.
9. Kept in protein block for 10 minutes.
10. Application of primary antibody (ER, PR, HER 2 neu) – 30 minutes.
11. Washed in TRIS wash buffer- 2 changes 10 minutes each.
12. Amplifier application for 15 minutes.
13. Washed in TRIS wash buffer- 2 changes 10 minutes each.
14. Application of secondary antibody (HRP POLYMERASE) – 20 minutes.

15. Washed in TRIS wash buffer- 2 changes 10 minutes each.
16. Application of Diamino-benzidine tetrachloride (DAB) chromogen 2 - 4 minutes.
17. Washed in distilled water – 2 changes.
18. Counterstaining is done with Hematoxylin for 30 seconds to impart background staining.
19. Wash in running tap water.
20. This is followed by dehydration, clearing and mounting.

**IMMUNOHISTOCHEMICAL EVALUATION**

Immuohistochemical analysis of a panel of HER2/neu, ER, PR were done in paraffin embedded tissue samples using polymer HRP system based on non-biotin polymeric technology. 5 μ thick sections from formalin fixed paraffin embedded tissue samples were transferred onto Poly-L-Lysine coated slides. Heat induced antigen retrieval was done. The antigen was bound with rabbit monoclonal antibody against HER2/neu, ER, PR and then detected by the addition of secondary antibody conjugated with horse radish peroxidase - polymer and diaminobenzidine substrate.

## TABLE 8: BIOMARKERS USED IN IHC

| Antigen | Species(clone) | Dilution |
|---|---|---|
| HER2/neu | Rabbit monoclonal | Ready to use |
| ER, PR | Rabbit monoclonal | Ready to use |

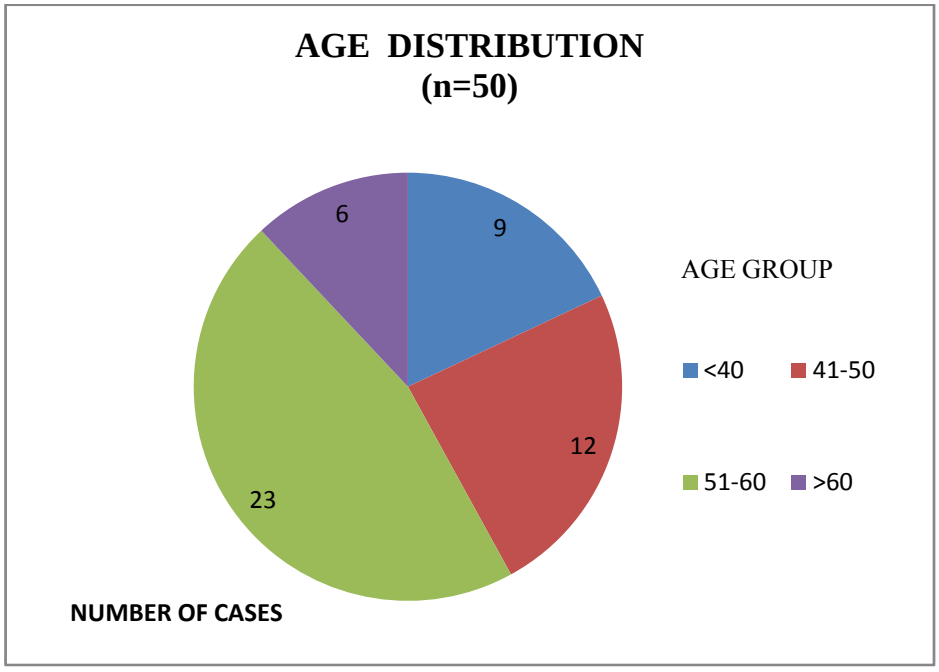
## OBSERVATION AND ANALYSIS

Of the 50 patients included in the study majority of the patients were between 50 and 60 years. Who constituted 46% of the group. The youngest patient was 33years old and the oldest patient is 75 years old.

**TABLE 9: AGE DISTRIBUTION OF PATIENTS**

| AGE (years) | NO. OF PATIENTS (n=50) | PERCENTAGE (%) |
|---|---|---|
| 31 – 40 | 9 | 18 |
| 41 – 50 | 12 | 24 |
| 51 – 60 | 23 | 46 |
| >60 | 6 | 12 |

**CHART 1: AGE DISTRIBUTION OF PATIENTS**



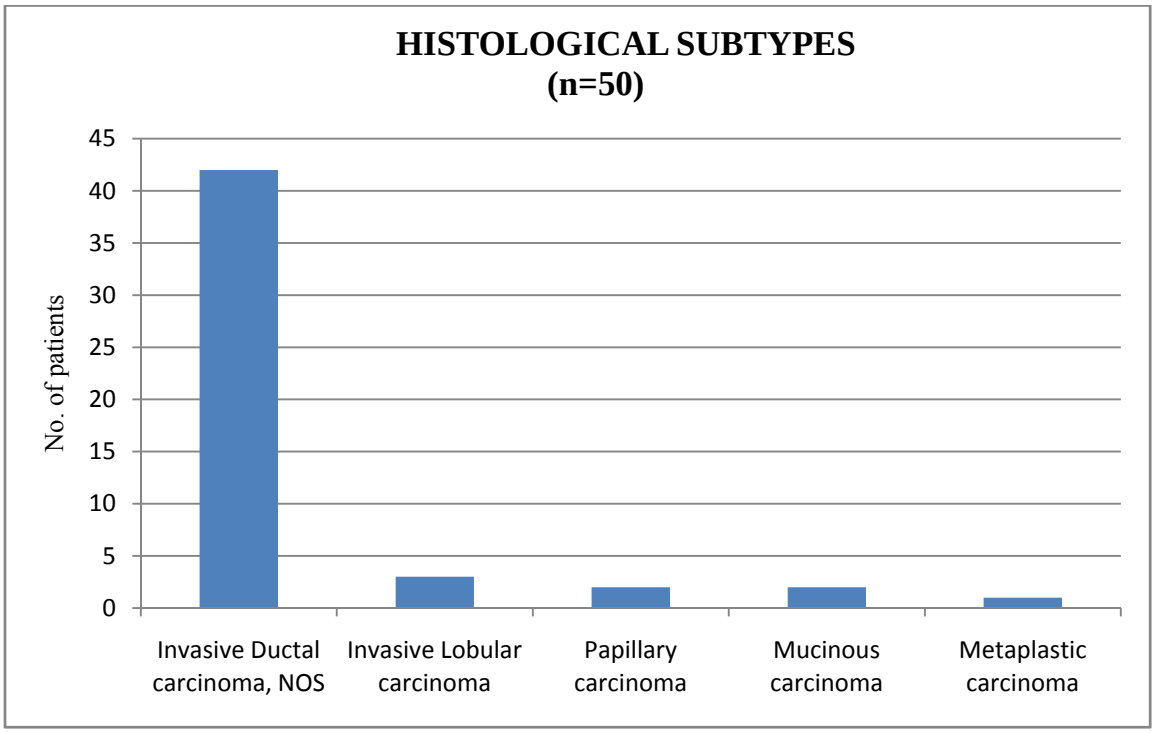
## DISTRIBUTION OF SAMPLES BASED ON HISTOLOGICAL SUBTYPES

Of the 50 patients analysed 42 (84%) were IDC, NOS. Invasive lobular carcinoma constituted 6% of the group followed by papillary and mucinous carcinoma each constitutes 4%. Metaplastic carcinoma was the least common type constituting 2% of the cases.

**TABLE 10: DISTRIBUTION OF SAMPLES BASED ON HISTOLOGICAL SUBTYPES**

| HISTOLOGICAL SUBTYPES | NO. OF PATIENTS(n=50) | PERCENTAGE (%) |
|---|---|---|
| Invasive Ductal carcinoma, NOS | 42 | 84 |
| Invasive Lobular carcinoma | 3 | 6 |
| Papillary carcinoma | 2 | 4 |
| Mucinous carcinoma | 2 | 4 |
| Metaplastic carcinoma | 1 | 2 |

## CHART 2: DISTRIBUTION OF SAMPLES BASED ON HISTOLOGICAL SUBTYPES



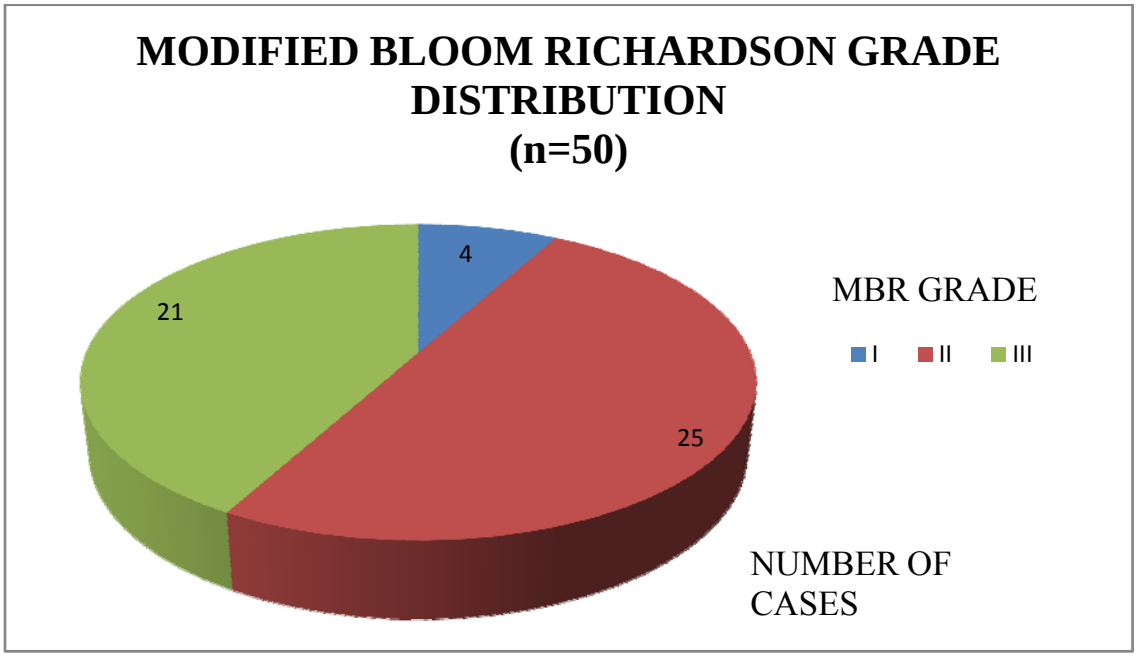

## DISTRIBUTION OF SAMPLE BASED ON MODIFIED BLOOM RICHARDSON GRADE

Modified Bloom Richardson scoring system was applied to all these cases. Among these cases, majority of the patients 25 (50%) were of MBR grade II followed 21 cases were grade III (42%) and 4 cases (8%) were under grade I.

**TABLE 11: DISTRIBUTION OF SAMPLES BASED ON MODIFIED BLOOM RICHARDSON GRADE**

| MBR GRADE | NO. OF PATIENTS (n=50) | PERCENTAGE (%) |
|---|---|---|
| I | 4 | 8 |
| II | 25 | 50 |
| III | 21 | 42 |

**CHART 3: DISTRIBUTION OF SAMPLES ACCORDING TO MODIFIED BLOOM RICHARDSON GRADE**



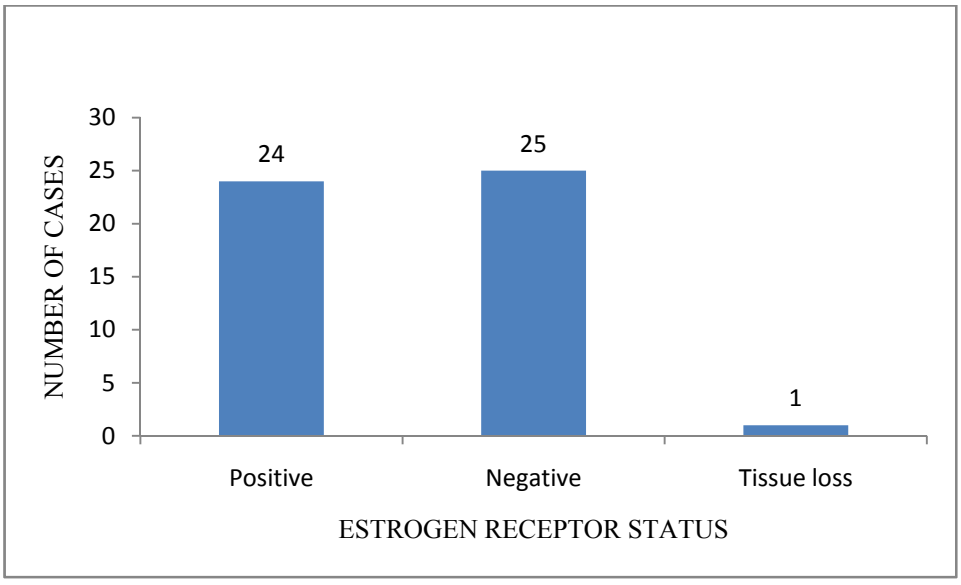
## DISTRIBUTION OF ER, PR, HER 2 / neu RECEPTORS:

There was an almost equal distribution of estrogen receptor positive (48%) and negative cases (50%), 2% of sample was lost during tissue processing. While analyzing the progesterone receptor status, majority of tumors were receptor positive (62%). Only 32% of cases did not express progesterone receptor and remaining 6% of samples were lost during tissue processing. 50% of the cases demonstrated HER-2/neu positivity and 40% of cases were negative. 10% of samples were lost during tissue processing.

**TABLE 12: DISTRIBUTION OF ER STATUS**

| ER STATUS | NO. OF PATIENTS (n=50) | Percentage (%) |
|---|---|---|
| Positive | 24 | 48 |
| Negative | 25 | 50 |
| Tissue loss | 1 | 2 |

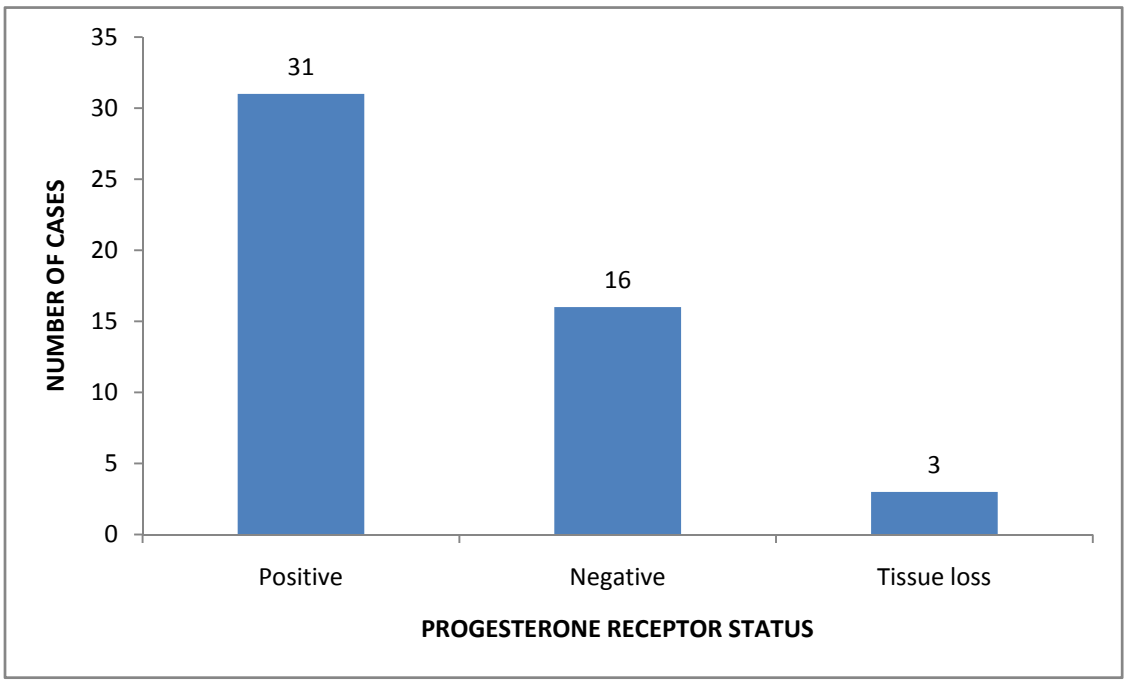
**CHART 4: DISTRIBUTION OF ER STATUS**



## TABLE 13: DISTRIBUTION OF PR STATUS

| PR STATUS | NO. OF PATIENTS (n=50) | Percentage (%) |
|---|---|---|
| Positive | 31 | 62 |
| Negative | 16 | 32 |
| Tissue loss | 3 | 6 |

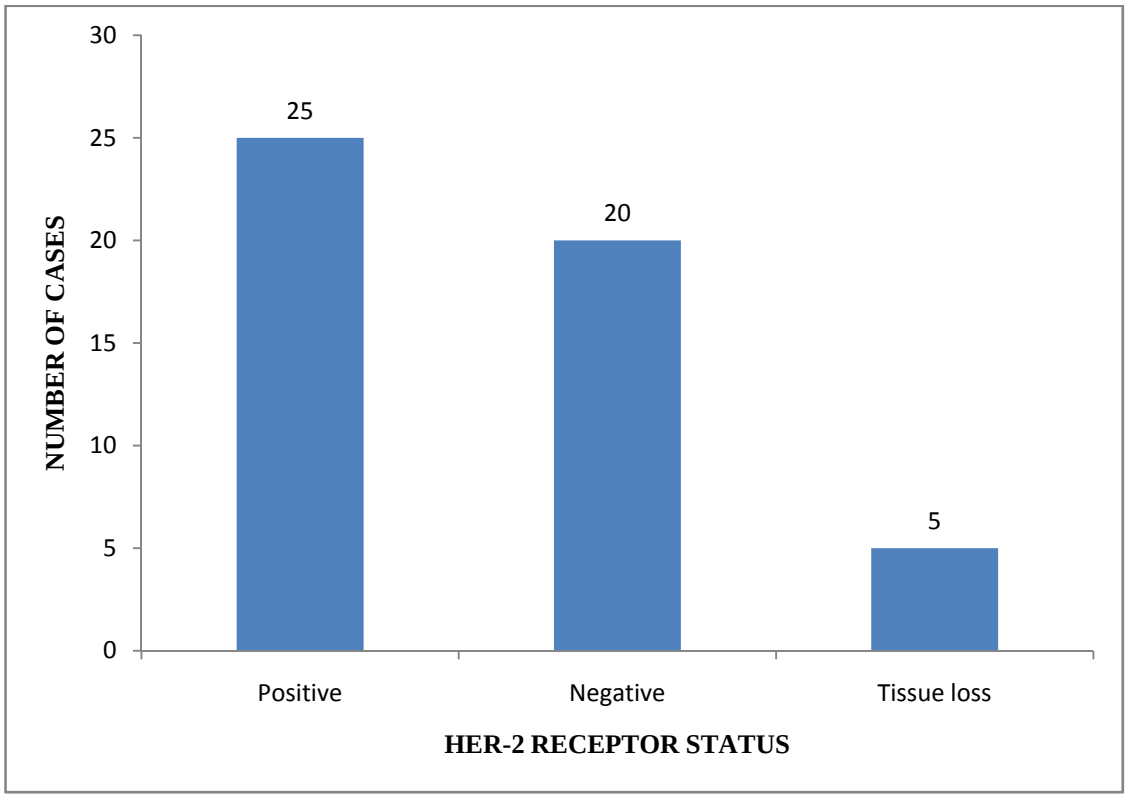
## CHART 5: DISTRIBUTION OF PR STATUS



## TABLE 14: DISTRIBUTION OF HER – 2/NEU STATUS

| HER -2 / neu STATUS | NO. OF PATIENTS (n=50) | Percentage (%) |
|---|---|---|
| Positive | 25 | 50 |
| Negative | 20 | 40 |
| Tissue loss | 5 | 10 |

## CHART 6: DISTRIBUTION OF HER – 2/NEU STATUS



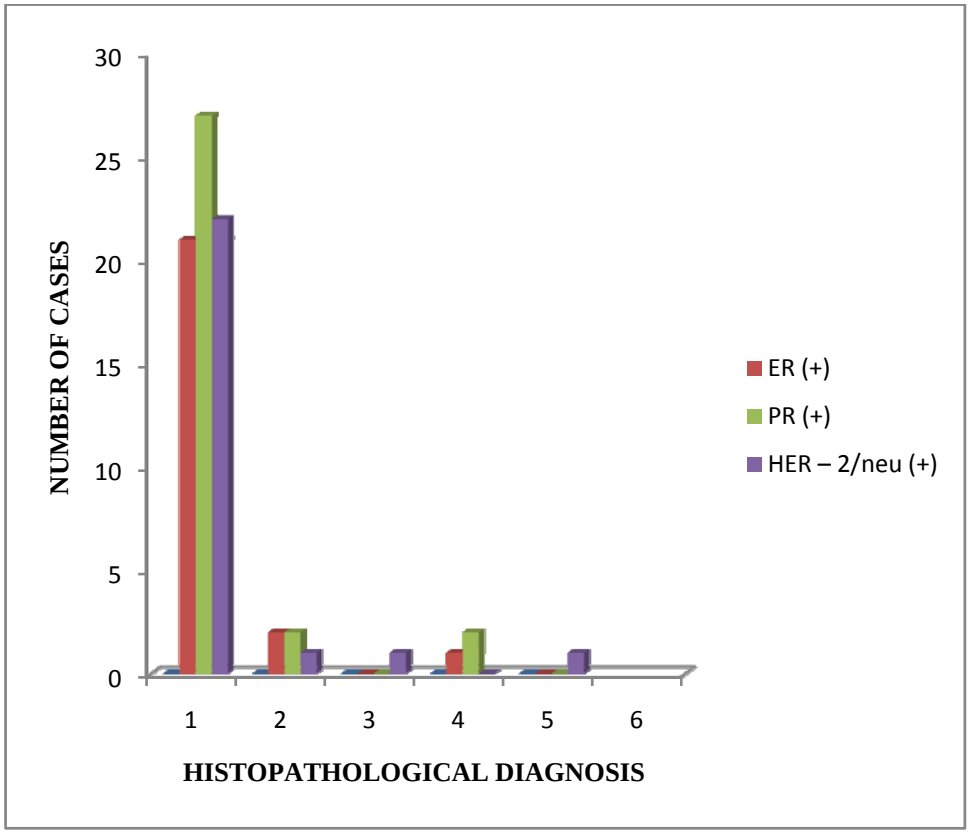
**CORRELATION BETWEEN HISTOLOGICAL SUBTYPES AND ER, PR AND HER – 2:**

Among cases of IDC, NOS type, almost 50% of cases showed ER positivity and HER 2/neu positivity. Around 60% of cases showed PR positivity. Among 3 cases of invasive Lobular carcinoma, 2 cases demonstrated ER and PR positivity and 1 case showed HER-2/neu positivity. Among 2 cases of papillary carcinoma, one case showed triple negative and the other case was ER and PR negative and HER-2 positive. None of the mucinous carcinoma cases showed HER-2neu positivity and the 1 case of metaplastic carcinoma was positive only for HER-2/neu.

**TABLE 15: RELATIONSHIP BETWEEN HISTOLOGIC SUBTYPES AND ER, PR AND HER-2/ NEU IN 50 CASES.**

| HPE DIAGNOSIS | ER (+) | PR (+) | HER – 2/neu (+) |
|---|---|---|---|
| IDC, NOS | 21 | 27 | 22 |
| Inv. Lobular ca | 2 | 2 | 1 |
| Papillary ca | 0 | 0 | 1 |
| Mucinous ca | 1 | 2 | 0 |
| Metaplastic ca | 0 | 0 | 1 |

## CHART 7: HISTOGRAM DEMONSTRATING RELATION BETWEEN HISTOLOGIC SUBTYPE AND RECEPTOR STATUS



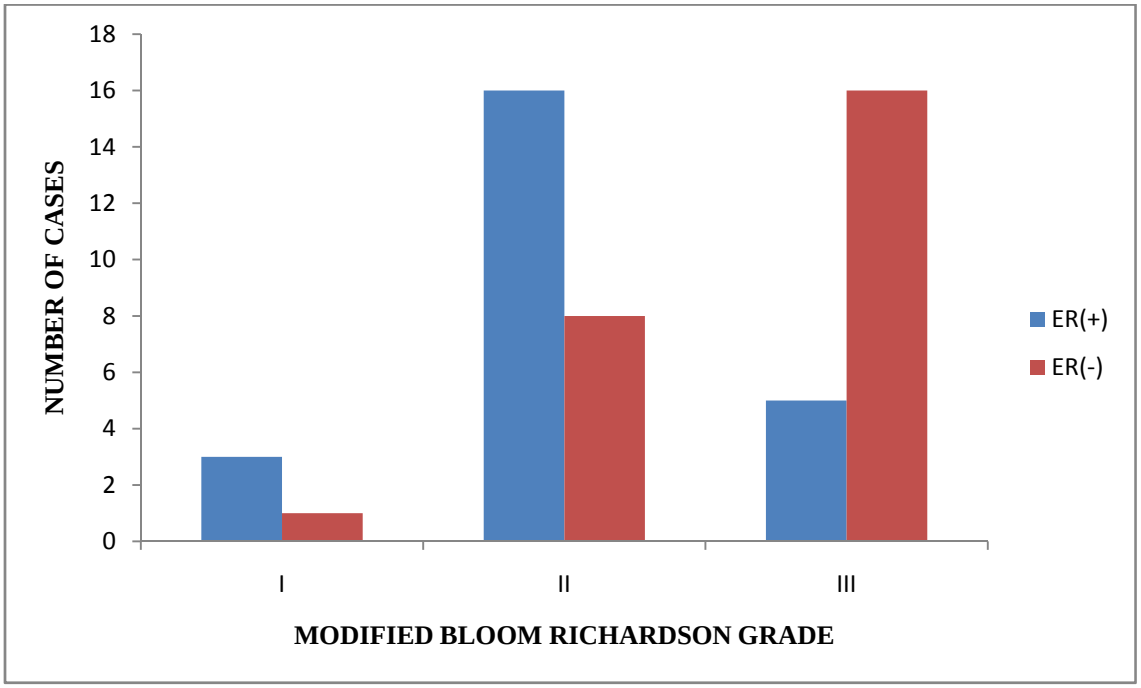

## CORRELATION OF ER STATUS WITH MODIFIED BLOOM RICHARDSON GRADE

Among Grade I tumors 3 were ER positive, 1 were ER negative. And among the 25 cases of Grade II tumors, 16 were ER positive and 8 ER negative. Total of 21 cases belonged to Grade III tumors, among them ER positive tumors were 5 and 16 were ER negative. The relationship between ER status and histological grading was assessed using Chi square test. It revealed a significant association, p value: 0.009.

## TABLE 16. RELATIONSHIP OF ER TO MODIFIED BLOOM RICHARDSON GRADE

| MBR GRADE | ER(+) | ER(-) | P value* Chi square test |
|---|---|---|---|
| I | 3 | 1 | 0.009 |
| II | 16 | 8 | |
| III | 5 | 16 | |

## CHART 8: HISTOGRAM SHOWING RELATIONSHIP BETWEEN ER STATUS AND MODIFIED BLOOM RICHARDSON GRADE.



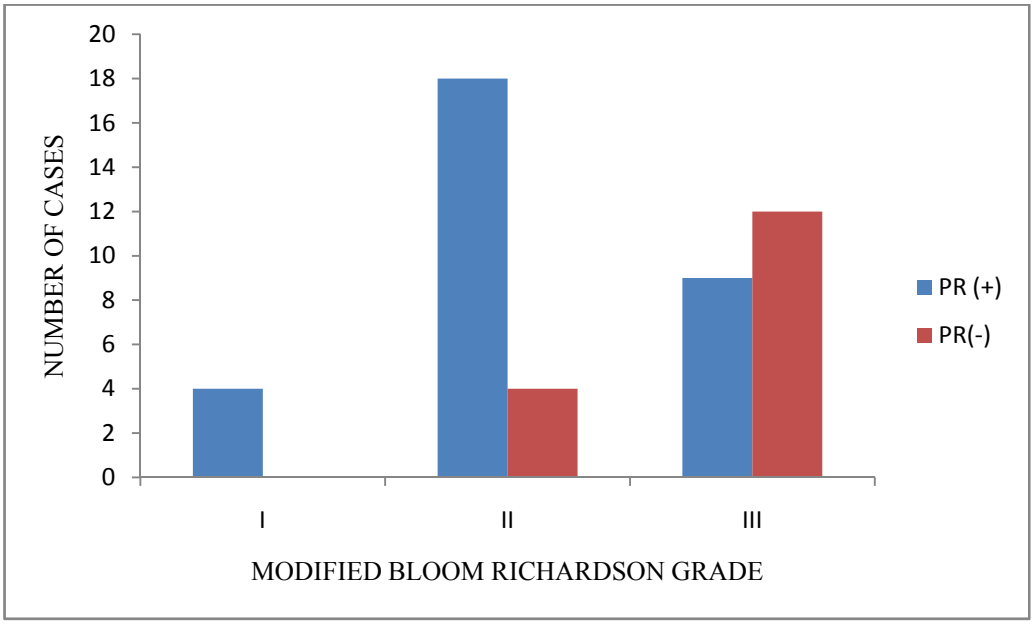
## CORRELATION OF PR WITH MODIFIED BLOOM RICHARDSON GRADE

All cases of grade I tumors were PR (+). Among the 25 cases of grade II tumors, 18 were PR (+), 4 were PR (-). Total of 21 cases of grade III tumors 9 were positive and 12 were negative. There was a significant statistical association between the progesterone receptor status and the histological grade (p value: 0.008, Chi square test).

**TABLE 17. RELATIONSHIP BETWEEN PR AND MODIFIED BLOOM RICHARDSON GRADE**

| GRADE | PR (+) | PR(-) | P value* Chi square test |
|---|---|---|---|
| I | 4 | 0 | 0.008 |
| II | 18 | 4 | |
| III | 9 | 12 | |

**CHART 9: HISTOGRAM SHOWING RELATIONSHIP BETWEEN PR STATUS AND MODIFIED BLOOM RICHARDSON GRADE.**




**CORRELATION BETWEEN HER 2/neu AND MODIFIED BLOOM RICHARDSON GRADE**

In the present study 14 HER 2(+) and 7 HER2 (-) was noted in grade II tumors. Among 21 grade III tumors 12 were positive and 9 were negative. The relationship between Her2neu status and histological grading was statistically significant (p value: 0.04) when assessed by Chi square test.

## TABLE 18: CORRELATION BETWEEN HER 2/neu AND MODIFIED BLOOM RICHARDSON GRADE

| GRADE | HER 2/neu (+) | HER 2/neu(-) | P value* Chi square test |
|---|---|---|---|
| I | 0 | 4 | 0.04 |
| II | 14 | 7 | |
| III | 12 | 9 | |

## CHART 10 : HISTOGRAM SHOWING RELATIONSHIP BETWEEN HER2 STATUS AND MODIFIED BLOOM RICHARDSON GRADE.

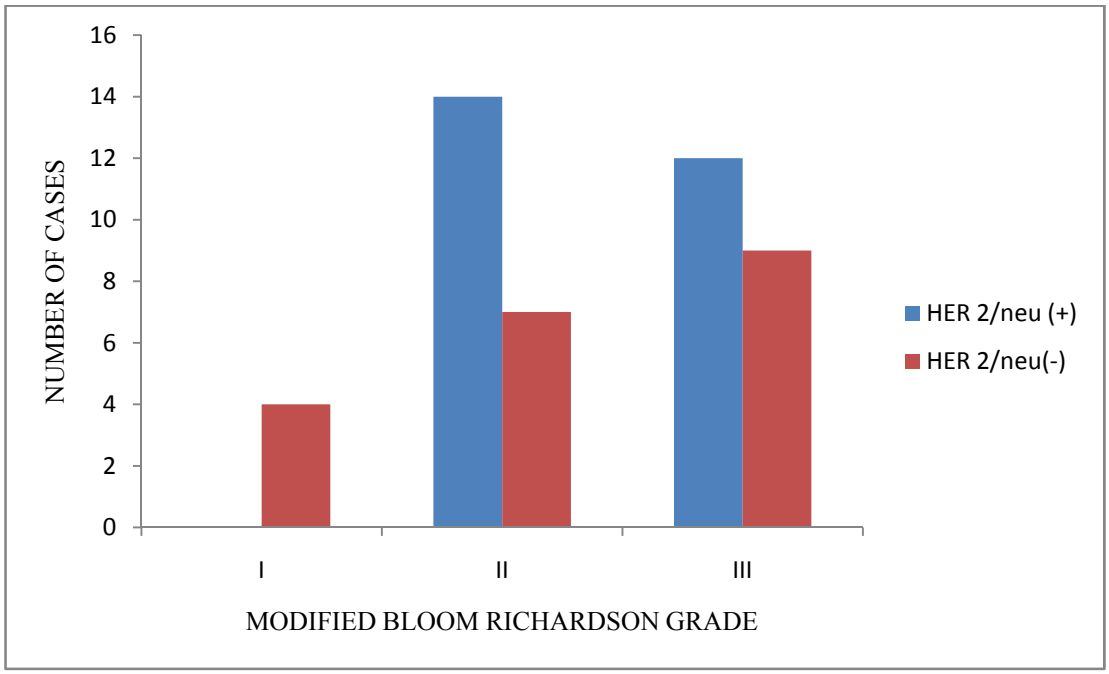


**TABLE 19: QUANTIFICATION OF IHC REAGENTS USED IN CONVENTIONAL VS TMA SECTIONS**

| IMMUNOHISTOCHEMICAL REAGENT | TMA | CONVENTIONAL | CONSUMPTION RATIO |
|---|---|---|---|
| Primary antibody | .11 IU | .8 IU | 1:7 |
| Secondary antibody | .11 IU | .8 IU | 1:7 |
| Chromogen | .11 IU | .8 IU | 1:7 |

The conventional immunohistochemistry using full section consumes 0.8 IU of the chemical reagents whereas only one seventh of the reagent is consumed by tissue microarray.

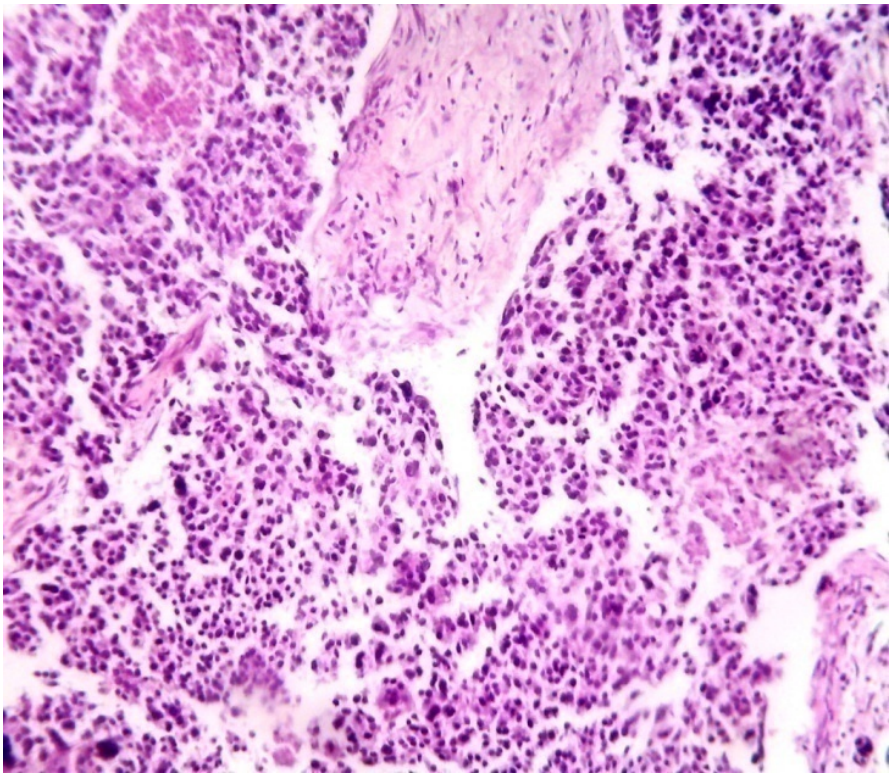


Fig.3 (A) – Invasive Ductal carcinoma (NOS) - Grade III (H&E, 100x)

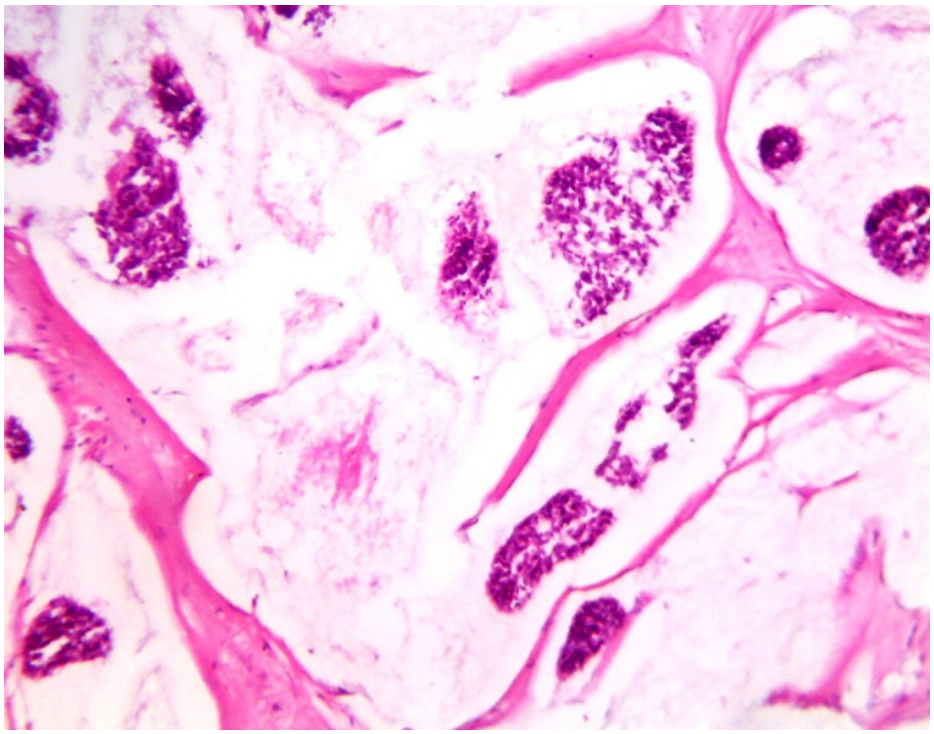


Fig. 3 (B) – Mucinous carcinoma (H & E, 100x)

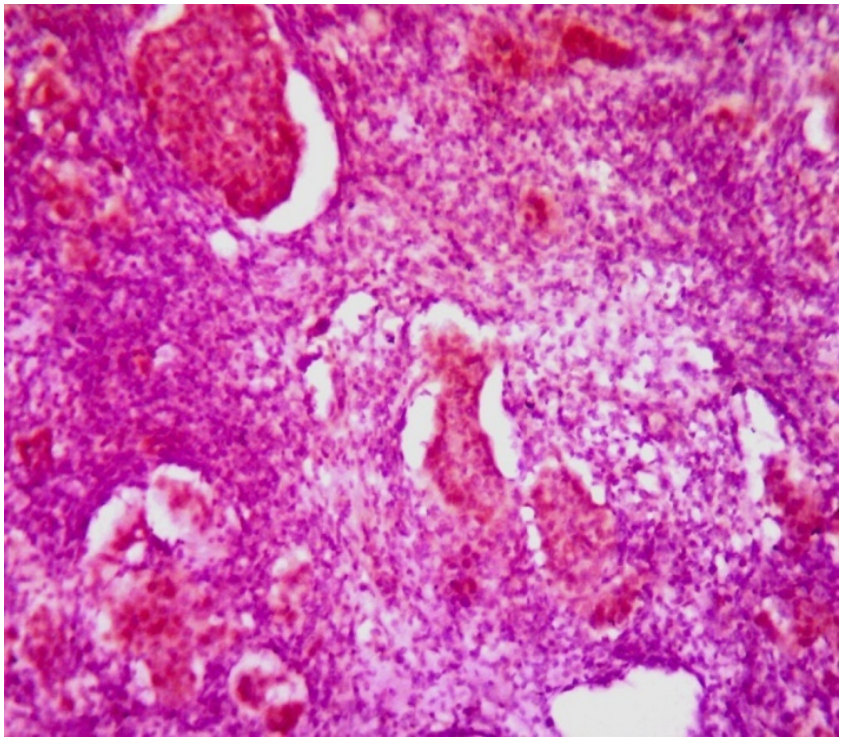


Fig. 4(A). Grade I IDC (NOS) - showing ER (+) of score 5 in the above tumor (IHC, 100x)

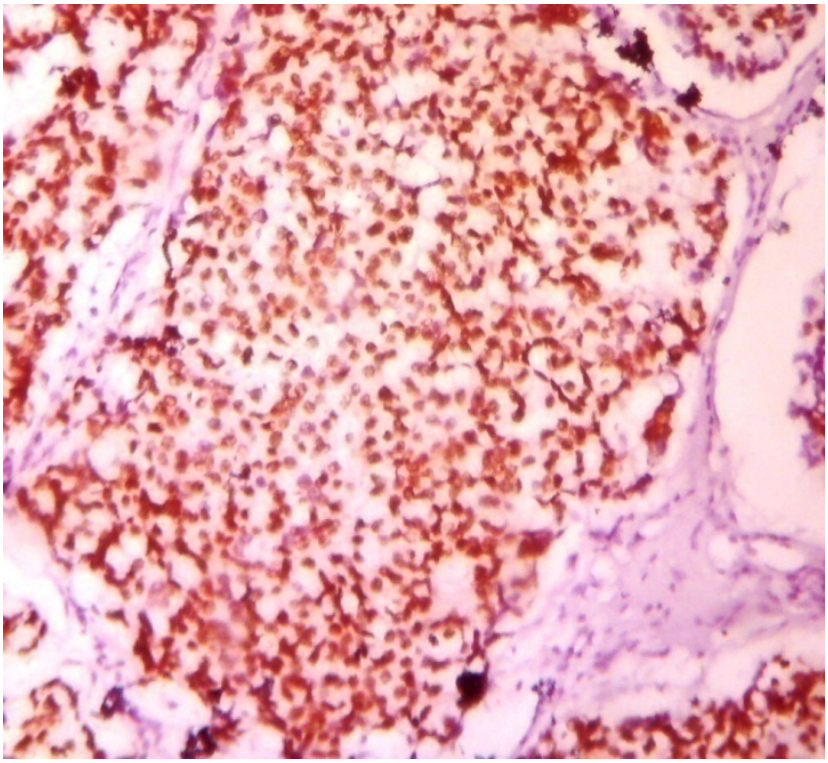


Fig. 4 (B) – Grade II IDC (NOS) showing intense PR (+) of score 8 in the above tumor (IHC, 100x)

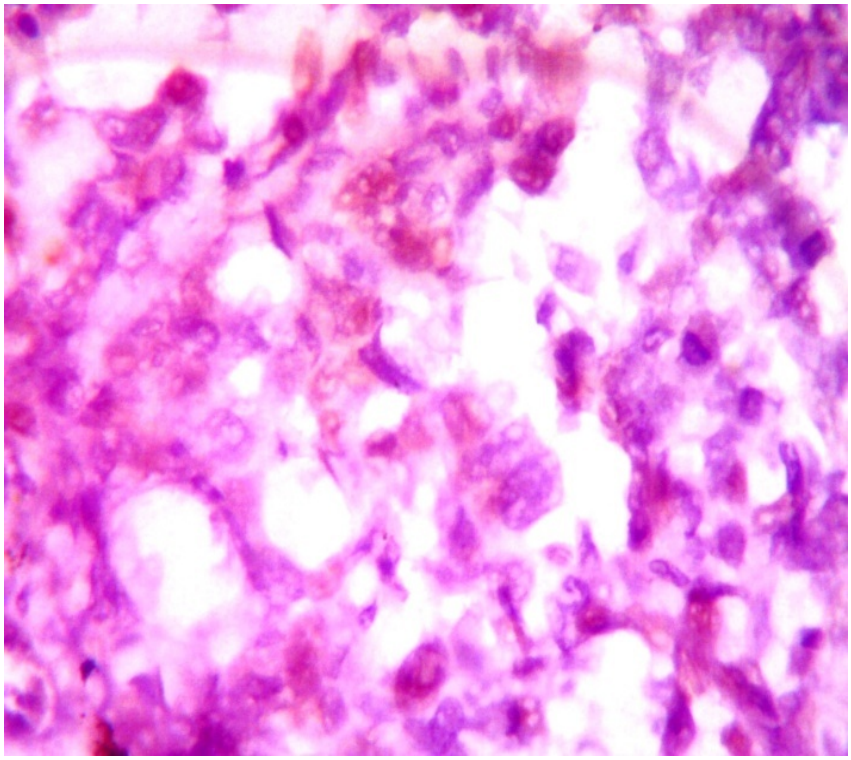


Fig. 4(C): Grade II IDC (NOS) –showing weak ER (+) of score 3 in the above tumor( IHC, 400x)

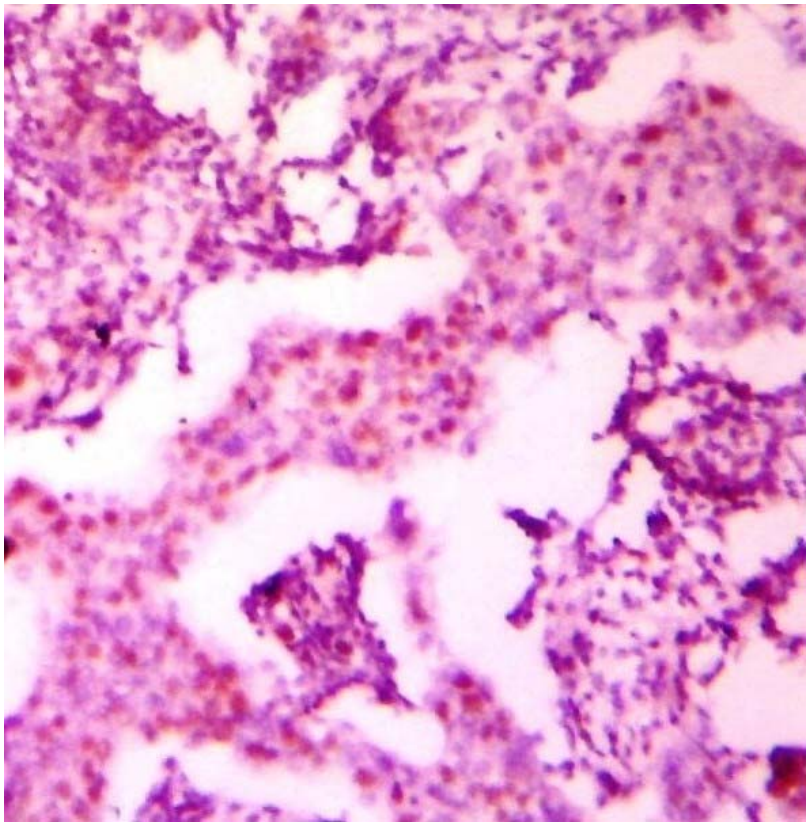


Fig. 5(A) – IDC (NOS) Grade II showing weak PR (+) of score 5 in the above tumor (IHC, 100x)

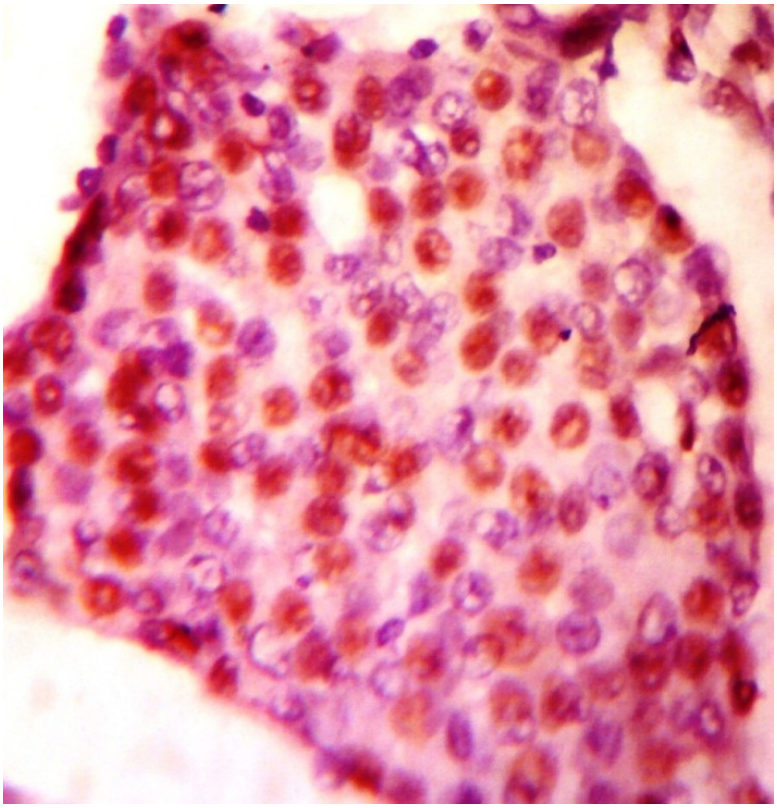


Fig. 5 (B) – Grade II IDC (NOS) showing PR (+) of score 8 in the above tumor (IHC, 400x)

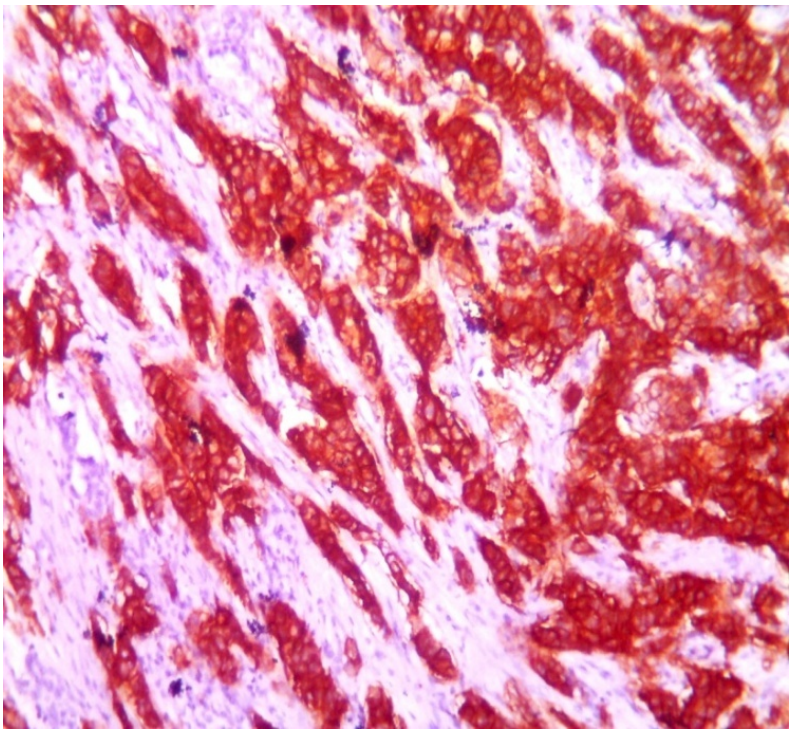


Fig. 6 (A) – Grade III IDC (NOS) showing strong HER 2 (+) of score 3 in the above tumor (IHC, 100x)

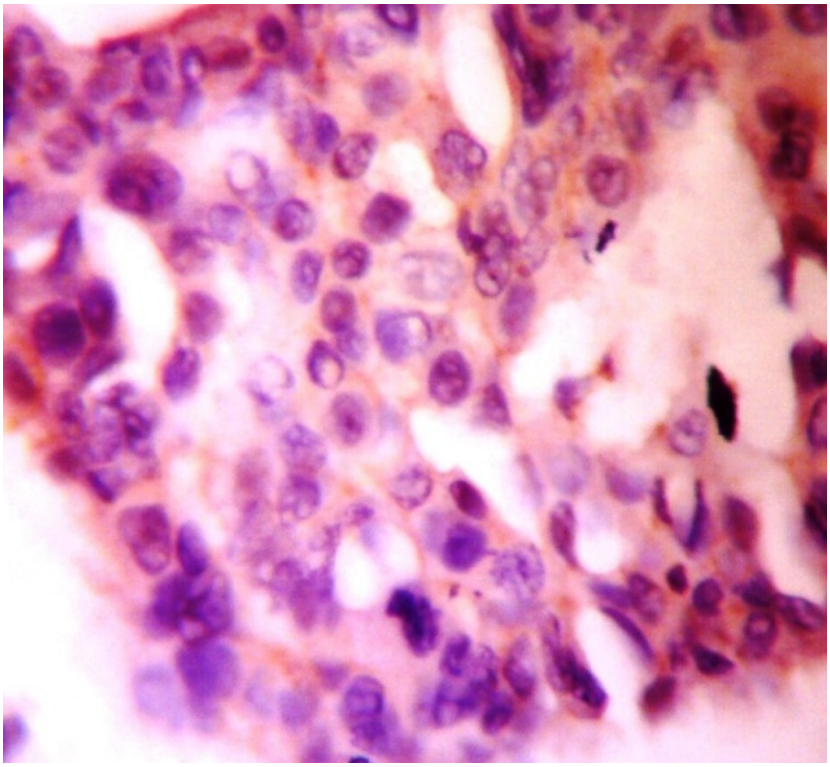


Fig. 6(B) – Grade II – IDC (NOS) showing weak HER 2 (+) of score 2 in the above tumor (IHC, 400x)

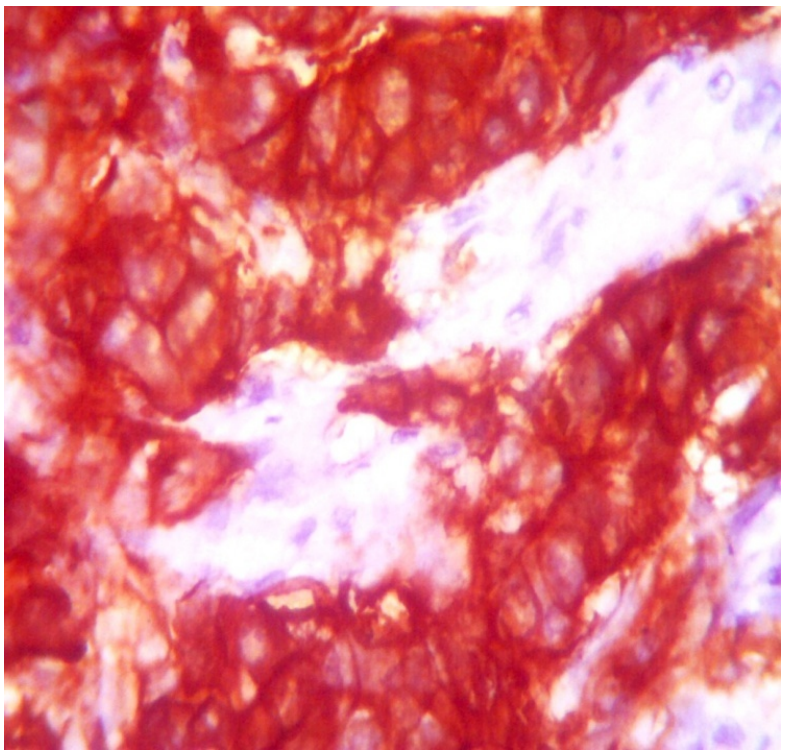


Fig. 6(C) – Grade III – IDC (NOS) showing intense HER 2 (+) of score 3 in the above tumor (IHC, 400x)

# DISCUSSION

The present study included 50 cases of histologically diagnosed Invasive breast carcinoma and Modified Bloom and Richardson grading system was applied for all these cases. Tissue Microarray was constructed for all these 50 cases on seven blocks and they were subjected to Immunohistochemical analysis to study ER, PR and HER 2 status in these tumors.

In the study done by Piero et al,[86] IDC (NOS) constituted 94.3% and ILC 5.7% of cases where as in Zafrani B et al study,[87] IDC (NOS) and ILC constituted 77% and 18% respectively. In the study conducted by Onitilo et al[88], IDC (NOS) was 72.7% and ILC was 12.1%. In the present study out of 50 cases, 42 were IDC (NOS) which constituted 84% and 3 were ILC which constituted 6% which is comparable to the previous studies quoted. Other subtypes of breast carcinomas had varied incidence in different studies and in the present study, remaining cases include papillary carcinoma (4%), mucinous carcinoma (4%) and metaplastic carcinoma (2%).

**TABLE 20: COMPARATIVE STUDY OF ER STATUS IN DIFFERENT TYPE OF BREAST CARCINOMA**

| ER positivity (%) | | | |
|---|---|---|---|
| | Lap P et al[89] | Ayadi et al[90] | Present study |
| IDC, NOS | 71.58% | 61.1% | 50% |
| ILC | 93.3% | 50% | 66.66% |
| Mucinous ca | 100% | 60% | 50% |
| Papillary ca | - | - | 0 |
| Metaplastic ca | 0 | - | 0 |

**TABLE 21: COMPARATIVE STUDY OF PR STATUS IN DIFFERENT TYPE OF BREAST CARCINOMA**

| PR positivity (%) | | | |
|---|---|---|---|
| | Lap P et al[89] | Ayadi et al[90] | Present study |
| IDC, NOS | 47.38% | 53.8% | 64.28% |
| ILC | 60.2% | 50% | 66.66% |
| Mucinous ca | 70% | 60% | 100% |
| Papillary ca | - | - | 0 |
| Metaplastic ca | 0 | - | 0 |

**TABLE 22: COMPARATIVE STUDY OF HER-2 STATUS IN DIFFERENT TYPE OF BREAST CARCINOMA**

| HER-2 / neu positivity (%) | | | |
|---|---|---|---|
| | Lap P et al[89] | Ayadi et al[90] | Present study |
| IDC, NOS | 17.54% | 16.8% | 52.38% |
| ILC | 0.8% | 16.7% | 33.33% |
| Mucinous ca | 0 | 0 | 0 |
| Papillary ca | - | - | 50% |
| Metaplastic ca | 0 | - | 100% |

In the study done by Lap P et al[89] and Ayadi et al[90] demonstrated high ER, PR positivity and low HER-2 expression with IDC (NOS), Invasive Lobular carcinoma and mucinous carcinoma. In the present study, IDC (NOS) showed high PR positivity but ER positivity was comparatively lower and HER-2 positivity was higher than above studies quoted whereas in Lobular carcinoma and Mucinous carcinoma, receptor status was almost equal to that of the studies quoted above (Table 20, 21 & 22). In the present study both the Papillary carcinoma was negative for ER and PR but one papillary carcinoma was positive for HER-2 which was similar to the above studies quoted. In the present study one case of Metaplastic carcinoma was negative for ER and PR, similar to the study of Lap P et al[89] (Table 20 & 21).

In the studies conducted by Zafrani et al,[87] Onitilo AA et al [88] and Piero G et al,[86] the most common tumors were grade II which constituted 40%,

38.4% and 37% respectively. In present study also majority were grade II tumors which constituted 50% of the cases.

In the study done by Ayadi et al [90], 72.2% of ER positive cases were grade I and II whereas 22.5% of ER (+) cases were grade III tumors. In the present study, out of 50 cases, 65.51% of ER positive cases were grade I and II, whereas 23.8% of ER (+) cases were grade III tumors. In the present study the relationship between ER expression and histological grading was statistically significant (p value: 0.009). In Ayadi et al[90] study, 61.4% of PR (+) cases were grade I and II tumors and 27.5% of PR (+) cases were grade III tumors. In the present study, 75.86% cases were grade I and II and 42.85% cases were grade III tumors (Table.23). In the present study, the relationship between PR expression and histological grading was statistically significant (p value: 0.008).

In the study done by Ayadi et al[90], 14.8% of HER 2 (+) cases were grade I and II tumors and 27.5% cases of HER 2 cases were grade III tumors whereas in the present study 48.27% of HER 2 (+) cases were in grade I and II and 57.14% cases of HER 2 (+) were in grade III (Table.23). In the present study the relationship between ER expression and histological grading was statistically significant (p value: 0.04).

**TABLE 23: COMPARISON OF STUDIES BASED ON ER, PR & HER 2 AND HISTOLOGICAL GRADES**

| | Ayadi et al[90] | | | Present study | | |
|---|---|---|---|---|---|---|
| Histological grade | ER (+) | PR (+) | HER 2(+) | ER(+) | PR(+) | HER2(+) |
| Grade I and II | 72.2% | 61.4% | 14.8% | 65.1% | 75.86% | 48.27% |
| Grade III | 22.5% | 27.5% | 27.5% | 23.8% | 42.85% | 57.14% |

Huang JH et al [91] and Ayadi L et al [90], have shown that grade I and grade II tumors showed increased expression of hormone receptors when compared to grade III tumors. In Ayadi et al, HER 2 expression was noted in 14.8% of grade I and II tumors whereas in the present study, HER 2 (+) cases was higher which constituted 48.27% (Table.23).

In the study done by Onitilo AA et al[88], overall triple negative cases were 13.4% whereas in the present study, triple negative cases were 10% of all cases.

According to Richter J et al [92], tissue loss due to technical problem ranged between 15-33%. D H Zhang et al[93], in his study observed tissue loss of 4% whereas in the present study overall tissue loss accounted to 10.66%.

To analyse single conventional tissue section immunohistochemically, minimum of two drops (0.8 IU) of chemical reagents which includes primary antibody, secondary antibody and DAB chromogen are required. Hence, a single tissue section when used conventionally consumes 0.8 IU of the reagent whereas in TMA, the same quantity has been used to analyze seven cores taken from seven different cases. So TMA, apart from having the advantage of parallel analysis of multiple sections, also decreases the time taken for the IHC procedure and the amount of chemical reagents used[92]. In this way, the use of immunohistochemical analysis in Invasive breast carcinoma by tissue microarray is helpful for economical and rapid study of receptor status.

# SUMMARY

This study was conducted in Department of Pathology, Tirunelveli Medical College from 50 cases of invasive breast carcinoma. Histological grading was done for all these cases according to Modified Bloom Richardson and the paraffin embedded tissue blocks from these cases were retrieved. Areas of invasive carcinoma were identified using corresponding haematoxylin and eosin stained slides and these areas were cored using bone marrow needle and transferred to recipient blocks. Thus Tissue microarray was constructed manually for 50 cases in 7 blocks. Array sections from these 7 blocks were obtained and they were subjected to immunohistochemical analysis using ER, PR and HER 2, of which 10.66% had tissue loss.

Among 50 cases, 42 cases (84%) were Invasive ductal carcinoma (NOS) type, 3 cases (6%) were Lobular carcinoma, 2 cases (4%) were Papillary, and 2 cases were (4%) Mucinous carcinoma and 1 (2%) Metaplastic carcinoma.

Among 50 cases, ER and PR were positive in 24 cases (48%) and 31 cases (62%) respectively. HER- 2 over expression was seen in 25 cases (50%).

Among the most common histologic subtype i.e., IDC (NOS), ER, PR and HER-2 were expressed in 50%, 64.28% and 52.38% respectively.

Among 50 cases, 4 cases (8%) of grade I tumors, 25 cases (50%) were under grade II tumors, and 21 cases (42%) were grade III.

Among the 50 cases, grade I and II tumors showed higher expression of hormone receptors whereas grade III tumors showed higher expression of HER-2.

Statistically significant values were noted between histological grade and ER, PR and HER-2 status.

# CONCLUSION

- The process of immunohistochemistry using conventional tissue section consumes more reagents, also requires control and standardisation for each batch which is not needed while using tissue microarray.
- Immunohistochemical analysis with a panel of markers using tissue microarray greatly reduces time and manual labour.
- By taking representative cores from different cases and performing IHC on them on a single slide proved to be economical.
- Tissue loss due to technical problems can be overcome by following standard protocols or by obtaining more number of tissue cores.

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## ANNEXURE-I

## WHO HISTOLOGICAL CLASSIFICATION OF TUMORS OF BREAST

**Epithelial tumors**

Invasive ductal carcinoma,not otherwise specified

- Mixed type carcinoma
- Pleomorphic carcinoma
- Carcinoma with osteoclast like giant cells
- Carcinoma with choriocarcinomatous features
- Carcinoma with melanotic features

Invasive lobular carcinoma

Tubular carcinoma

Invasive cribriform carcinoma

Medullary carcinoma

Mucinous carcinoma and other tumors with abundant mucin

- Mucinous carcinoma
- Cystadenocarcinoma and columnar cell mucinous carcinoma
- Signet ring carcinoma

Neuroendocrine tumors

- Solid neuroendocrine carcinoma
- Atypical carcinoid tumor
- Small cell/oat cell carcinoma
- Large cell neuroendocrine carcinoma

Invasive papillary carcinoma

Invasive micropapillary carcinoma

Apocrine carcinoma

Metaplastic carcinoma

- Pure epithelial metaplastic carcinoma
- Squamous cell carcinoma
- Adeno carcinoma with spindle cell metaplasia
- Adenosquamous carcinoma
- Mucoepidermoid carcinoma
- Mixed epithelial/mesenchymal metaplastic carcinoma

Lipid rich carcinoma

Secretory carcinoma

Oncocytic carcinoma

Adenoid cystic carcinoma

Acinic cell carcinoma

Glycogen rich clear cell carcinoma

Sebaceous carcinoma

Inflammatory carcinoma

Lobular neoplasia

- Lobular carcinoma in situ

Intraductal proliferative lesions

- Usual ductal hyperplasia
- Flat epithelial atypia

- Atypical ductal hyperplasia
- Ductal carcinoma on situ

Microinvasive carcinoma

Intraductal papillary neoplasm

- Central papilloma
- Peripheral papilloma
- Atypical papilloma
- Intraductal papillary carcinoma
- Intracystic papillary carcinoma

Adenosis including variants

- Sclerosing Adenosis
- Apocrine Adenosis
- Blunt duct Adenosis
- Microglandular Adenosis
- Adenomyoepithelial Adenosis

Radial scar/complex sclerosing lesion

**Adenomas**

- Tubular Adenoma
- Lactating Adenoma
- Apocrine Adenoma
- Pleomorphic Adenoma
- Ductal Adenoma

**Myoepithelial lesions**

- Myoepitheliosis
- Adenomyoepithelial adenosis
- Adenomyoepithelioma
- Malignant myoepithelioma

**Mesenchymal tumors**

- Hemangioma
- Angiomatosis
- Hemangiopericytoma
- Pseudoangiomatous stromal hyperplasia
- Myofibroblastoma
- Fibromatosis
- Inflammatory myofibroblastic tumor
- Lipoma
- Angiolipoma
- Granular cell tumor
- Neurofibroma
- Schwannoma
- Angiosarcoma
- Liposarcoma
- Rhabdomyosarcoma
- Osteosarcoma
- Leiomyoma

Leiomyosarcoma

**Fibroepithelial tumors**

Fibroadenoma

Phyllodes tumor

- Benign
- Borderline
- Malignant

Periductal stromal sarcoma,low grade

Mammary hamartoma

**Tumors of the nipple**

Nipple adenoma

Syringomatous adenoma

Pagets disease of nipple

**Malignant lymphoma**

Diffuse large B cell lymphoma

Burkitt's lymphoma

Extranodal marginal zone B Cell lymphoma

Follicular lymphoma

**Metastatic tumors**

**Tumors of the male breast**

Gynaecomastia , Carcinoma :invasive, insitu

## ANNEXURE 2

## PROCESSING FOR IMMUNOHISTOCHEMISTRY

- Section cutting and incubation is followed by Xylene wash (2 changes) for 10 minutes each.
- Rehydrated in graded alchohol containing 100%, 80%, 70% for ten minutes each.
- Rinsed in distilled water for 2 minutes.
- Antigen retrieval.
- Cooling for 15 minutes.
- Washed in TRIS wash buffer- 2 changes 5 minutes each.
- Treated with peroxide block for 5 minutes.
- Washed in TRIS wash buffer- 2 changes 10 minutes each.
- Kept in protein block for 10 minutes.
- Application of primary antibody (ER, PR, HER 2 neu) – 30 minutes.
- Washed in TRIS wash buffer- 2 changes 10 minutes each.
- Amplifier application for 15 minutes.
- Washed in TRIS wash buffer- 2 changes 10 minutes each.
- Application of secondary antibody (HRP POLYMERASE) – 20 minutes.
- Washed in TRIS wash buffer- 2 changes 10 minutes each.
- Application of Diamino-benzidine tetrachloride (DAB) chromogen 2 - 4 minutes.
- Washed in distilled water – 2 changes.

- Counterstaining is done with Hematoxylin for 30 seconds to impart background staining.
- Wash in running tap water.
- This is followed by dehydration, clearing and mounting.

**BUFFER PREPARATIONS**

**1) Tris – EDTA Buffer: - PH- 9.0**

| | |
|---|---|
| Tris | - 6.05 gm |
| EDTA | - 0.744 gm |
| Distilled water | - 1000 ml |

**2) Citrate buffer :- p H-6.2**

| | |
|---|---|
| Citrate | - 1.92 gm |
| Distilled water | -1000 ml |

**3) Tris wash buffer**

| | |
|---|---|
| Tris | - 0.605 gm |
| Sodium chloride | - 8 gm |
| 1 N Hcl | - 4ml |
| Distilled water | - 1000 ml |

**PRECAUTIONS**

1. The glasswares used should be dry and clean.
2. All the buffers used should be prepared fresh and the pH should be adjusted according to the preferred pH.
3. The staining procedures are never allowed to dry so they are performed under a humidity chamber.
4. DAB chromogen should be handled and disposed carefully as it is a carcinogen.
5. Primary, secondary antibody, DAB chromogen, peroxidase block, amplifier , everything should be stored at 4-6ºC

## ANNEXURE 3

## ALLRED SCORING GUIDELINES FOR ER AND PR

**Proportion score**

Proportion score is done by calculating the proportion of tumor cells with positive nuclear staining

0 = no nuclear staining

1 = <1% nuclear staining

2 = 1%-10% nuclear staining,

3 = 11%-33% nuclear staining

4 = 34%-66% nuclear staining

5 = 67%-100% nuclear staining

**Intensity of staining**

0 = no staining

1 = weak staining

2 = moderate staining

3 = strong staining

**Total score =proportion score + intensity score (0 to 8)**

Interpretation:.

0,2 - Negative

≥ 3 - Positive

**GRADING OF THE IMMUNOHISTOCHEMICAL STAINING FOR HER 2 / NEU OVEREXPRESSION**

| Score | Staining Pattern | HER 2 / neu protein overexpression assessment |
|---|---|---|
| **0** | No staining at all or very slight partial membrane staining in less than 10% of tumor cells. | Negative |
| **1+** | Faint barely perceptible membrane staining in more than 10% of tumor cells. Cells stained in only part of the membrane. | Negative |
| **2+** | Weak to moderate complete membrane staining observed in more than 10% of tumor cells. | Weakly Positive |
| **3+** | Strong complete membrane staining in more than 30% of tumor cells | Strongly Positive |

# MASTER CHART

| S.NO | HPE NO | AGE | SEX | HISTOPATHOLOGICAL DIAGNOSIS | MBR GRADING | ER STATUS BY ALLRED SCORING SYSTEM | | | PR STATUS BY ALLRED SCORING SYSTEM | | | HER 2/ NEU BY ASCO SCORING SYSTEM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | PROPORTION | INTENSITY | SCORE | PROPORTION | INTENSITY | SCORE | |
| 1 | 391/ 15 | 60 | F | IDC, NOS | II | 5 | 2 | Positive | 3 | 2 | Positive | Positive |
| 2 | 400/ 15 | 36 | F | IDC, NOS | II | 4 | 2 | Positive | 3 | 2 | Positive | Positive |
| 3 | 271 /15 | 67 | F | IDC, NOS | III | 3 | 2 | Positive | 4 | 2 | Positive | Positive |
| 4 | 541/ 15 | 39 | F | IDC, NOS | III | 2 | 2 | Positive | 2 | 1 | Positive | Negative |
| 5 | 1238/ 15 | 45 | F | IDC, NOS | II | 5 | 2 | Positive | 5 | 2 | Positive | Positive |
| 6 | 139/ 15 | 36 | F | IDC, NOS | II | 3 | 2 | Positive | 3 | 2 | Positive | Negative |
| 7 | 198/ 15 | 60 | F | IDC, NOS | I | 3 | 1 | Positive | 2 | 2 | Positive | Negative |
| 8 | 2328/ 14 | 50 | F | Papillary carcinoma | III | 0 | 0 | Negative | 0 | 0 | Negative | Positive |
| 9 | 1717/ 15 | 52 | F | IDC, NOS | III | 0 | 0 | Negative | 0 | 0 | Negative | Positive |
| 10 | 1510/ 15 | 60 | F | Metaplastic carcinoma | III | 0 | 0 | Negative | 0 | 0 | Negative | Positive |
| 11 | 1075/ 15 | 56 | F | IDC, NOS | II | 0 | 0 | Negative | 2 | 2 | Positive | Positive |
| 12 | 1176/ 15 | 63 | F | IDC, NOS | III | 0 | 0 | Negative | 2 | 2 | Positive | Positive |
| 13 | 1741/ 15 | 57 | F | IDC, NOS | II | 0 | 0 | Negative | 2 | 1 | Positive | Positive |
| 14 | 1760/ 15 | 50 | F | IDC, NOS | III | 0 | 0 | Negative | 0 | 0 | Negative | Negative |
| 15 | 1629/ 14 | 60 | F | IDC, NOS | II | 4 | 2 | Positive | 2 | 1 | Positive | Negative |
| 16 | 1820/ 15 | 35 | F | Mucinous carcinoma | I | 3 | 2 | Positive | 4 | 3 | Positive | Negative |
| 17 | 3184/ 14 | 53 | F | IDC, NOS | II | 3 | 1 | Positive | 3 | 3 | Positive | Positive |
| 18 | 1820/ 14 | 35 | F | IDC, NOS | II | 0 | 0 | Negative | 0 | 0 | Negative | Positive |
| 19 | 2227/ 14 | 60 | F | IDC, NOS | II | 0 | 0 | Negative | 0 | 0 | Negative | Positive |
| 20 | 1637/ 14 | 50 | F | IDC, NOS | II | 5 | 2 | Positive | 4 | 2 | Positive | Negative |
| 21 | 2411/ 14 | 45 | F | IDC, NOS | II | 0 | 0 | Negative | 0 | 0 | Negative | Positive |
| 22 | 2053/ 14 | 41 | F | Invasive Lobular carcinoma | II | | | Tissue Loss | | | Tissue Loss | Tissue loss |
| 23 | 244/ 15 | 49 | F | IDC, NOS | III | 0 | 0 | Negative | 0 | 0 | Negative | Negative |
| 24 | 3264/ 14 | 52 | F | Mucinous carcinoma | I | 0 | 0 | Negative | 2 | 1 | Positive | Negative |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 25 | 1651/ 14 | 65 | F | Papillary carcinoma | III | 0 | 0 | Negative | 2 | 1 | Negative | Negative |
| 26 | 858/ 14 | 61 | F | IDC, NOS | III | 0 | 0 | Negative | 2 | 1 | Positive | Positive |
| 27 | 615/ 15 | 58 | F | IDC, NOS | II | 0 | 0 | Negative | | | Tissue Loss | Tissue loss |
| 28 | 977/ 14 | 46 | F | IDC, NOS | II | 3 | 1 | Positive | 2 | 1 | Positive | Negative |
| 29 | 1616/ 15 | 33 | F | IDC, NOS | II | 2 | 1 | Positive | | | Tissue Loss | Tissue loss |
| 30 | 614/ 15 | 40 | F | IDC, NOS | II | 4 | 3 | Positive | 3 | 2 | Positive | Negative |
| 31 | 762/ 15 | 55 | F | Invasive Lobular carcinoma | II | 5 | 3 | Positive | 2 | 2 | Positive | Positive |
| 32 | 829/ 15 | 57 | F | IDC, NOS | III | 0 | 0 | Negative | 0 | 0 | Negative | Positive |
| 33 | 726/ 15 | 44 | F | IDC, NOS | II | 0 | 0 | Negative | 0 | 0 | Negative | Positive |
| 34 | 854/ 14 | 58 | F | IDC, NOS | III | 0 | 0 | Negative | 0 | 0 | Negative | Positive |
| 35 | 494/ 14 | 64 | F | IDC, NOS | III | 0 | 0 | Negative | 0 | 0 | Negative | Negative |
| 36 | 2067/ 14 | 45 | F | IDC, NOS | II | 2 | 2 | Positive | 2 | 1 | Positive | Positive |
| 37 | 196/ 15 | 40 | F | IDC, NOS | II | 0 | 0 | Negative | 2 | 1 | Positive | Positive |
| 38 | 345/ 15 | 39 | F | IDC, NOS | III | 5 | 3 | Positive | 2 | 1 | Positive | Negative |
| 39 | 124/ 15 | 60 | F | IDC, NOS | III | 4 | 3 | Positive | 0 | 0 | Negative | Negative |
| 40 | 199/ 15 | 55 | F | IDC, NOS | II | 3 | 1 | Positive | 2 | 1 | Positive | Negative |
| 41 | 153/ 15 | 75 | F | IDC, NOS | III | 0 | 0 | Negative | 3 | 2 | Positive | Positive |
| 42 | 106/ 15 | 36 | F | IDC, NOS | III | 0 | 0 | Negative | 0 | 0 | Negative | Negative |
| 43 | 2608/ 14 | 57 | F | IDC, NOS | III | 3 | 2 | Positive | 3 | 2 | Positive | Tissue loss |
| 44 | 2979/ 14 | 53 | F | IDC, NOS | III | 0 | 0 | Negative | 0 | 0 | Negative | Positive |
| 45 | 2850/ 14 | 60 | F | IDC, NOS | III | 0 | 0 | Negative | 4 | 2 | Positive | Positive |
| 46 | 3137/ 14 | 55 | F | IDC, NOS | II | 4 | 2 | Positive | 3 | 2 | Positive | Tissue loss |
| 47 | 2851/ 14 | 43 | F | IDC, NOS | I | 5 | 3 | Positive | 4 | 2 | Positive | Negative |
| 48 | 2666/ 14 | 54 | F | Invasive Lobular carcinoma | II | 3 | 1 | Positive | 5 | 3 | Positive | Negative |
| 49 | 3315/ 14 | 60 | F | IDC, NOS | III | 0 | 0 | Negative | 5 | 2 | Positive | Negative |
| 50 | 3119/ 14 | 42 | F | IDC, NOS | II | 5 | 2 | Positive | 4 | 2 | Positive | Positive |